Charakterisierung der methanotrophen Lebensgemeinschaften im Reisfeld- und Waldboden

DISSERTATION

zur
Erlangung des Doktorgrades der Naturwissenschaften
(Dr. rer. nat.)

dem Fachbereich Biologie der
Philipps-Universität Marburg/Lahn
vorgelegt von

Thilo Hans Eckhardt Henckel
aus Hannover

Marburg/Lahn 2000

Die Untersuchungen zur vorliegenden Arbeit wurden von Mai 1997 bis Dezember 1999 am Max-Planck-Institut für terrestrische Mikrobiologie in Marburg/Lahn unter der Leitung von Professor Dr. Ralf Conrad durchgeführt.

Vom Fachbereich Biologie der Philipps-Universität Marburg als Dissertation angenommen am:

Referent: Prof. Dr. R. Conrad

Korreferent. Prof. Dr. R. Thauer

Tag der Disputation:

Die in dieser Dissertation beschriebenen Ergebnisse sind in folgenden Originalpublikationen veröffentlicht bzw. zur Veröffentlichung eingereicht:

Dunfield, P., F., Liesack, W., Henckel, T., Knowles, R., Conrad, R. (1999) High-affinity methane oxidation by a soil enrichment culture containing a type II methanotroph, *Appl. Environ. Microbiol*., 65, 1009-1014.

Henckel, T., Friedrich, M. and Conrad, R. (1999) Molecular analyses of the methane-oxidizing microbial community in rice field soil by targeting the genes of the 16S rRNA, particulate methane monooxygenase, and methanol dehydrogenase, *Appl. Environ. Microbiol*., 65, 1980-1990.

Bodelier, P. L. E., Roslev, P., Henckel, T., and Frenzel, P. (2000) Stimulation by ammonium-based fertilisers of methane oxidation in soil around rice roots, *Nature*, 403, 421-424.

Henckel, T., Jäckel, U., Schnell, S., and Conrad, R. (2000) Molecular analyses of novel methanotrophic communities in forest soil that oxidize atmospheric methane, *Appl. Environ. Microbiol*., 66, pp. 1801-1808.

Henckel, T., Roslev, P., and Conrad, R. (submitted) Effects of O_2 and CH_4 on presence and activity of the indigenous methanotrophic community in rice field soil. *Environmental Microbiology*

Henckel, T., Jäckel, U., and Conrad, R. (submitted) Vertical distribution of the methanotrophic comunity after drainage of rice field soil. *Fems Microbiol. Ecology*.

Inhaltsverzeichnis

Abkürzungsverzeichnis

DGGE	Denaturierende-Gradienten-Gel-Elektrophorese
GC-Klammer	GC-reiche-Nukleotidsequenz am 5´-Ende eines Primers zur Amplifikation von PCR-Produkten für die DGGE-Analyse
gdw	Trockengewicht in Gramm ("gram dryweight")
gFG	Feuchtgewicht in Gramm
gfw	Feuchtgewicht in Gramm ("gram freshweight")
gTG	Trockengewicht in Gramm
MB10γ	Oligonukleotid-Primerpaar zur Amplifikation eines 16S rRNA-Genabschnittes, bindet an Regionen spezifisch für methylotrophe γ-Proteobakterien
MB9α	Oligonukleotid-Primerpaar zur Amplifikation eines 16S rRNA-Genabschnittes, bindet an Regionen spezifisch für methylotrophe α-Proteobakterien
MPN	"most probable number"
mxaF	Oligonukleotid-Primerpaar zur Amplifikation des *mxa*F-Gens der Methanol-Dehydrogenase
PLFA	Phospholipide ("phospholipid fatty acids")
PCR	Polymerase-Kettenreaktion ("polymerase chain reaction")
pmoA	Oligonukleotid-Primerpaar zur Amplifikation des *pmo*A-Gens der partikulären Methan-Monooxygenase
ppmv	Mischungsverhältnis: "parts per million by volume"
sp.	Art (Spezies)
syn. Luft	synthetische Luft (20,5 % O_2 in 79,5 % N_2)
Temed	N,N,N´,N´-Tetramethylethylendiamin
TGGE	Temperatur-Gradienten-Gel-Elektrophorese
"Universal"	Oligonukleotid-Primerpaar zur Amplifikation eines 16S rRNA-Genabschnittes, bindet an Regionen vertreten in *Bacteria* und *Archaea*
v/v	Volumen pro Volumen
wt/wt	Gewicht pro Gewicht ("weight by weight")

Weitere Abkürzungen wurden entsprechend der "Information for Authors" des *European Journal of Biochemistry* verwendet.

I. Zusammenfassung

Die mikrobielle Methanoxidation vermindert die Freisetzung des Treibhausgases Methan (CH_4) aus wassergesättigten, anoxischen Böden in die Atmosphäre. Außerdem ermöglicht sie die Aufnahme von atmosphärischem CH_4 in oxischen Böden. In der vorliegenden Arbeit wurden die dafür verantwortlichen Lebensgemeinschaften der methanoxidierenden Bakterien (MOB) in einem italienischen Reisfeldboden und einem sauren Waldboden charakterisiert.

Die methanotrophen Lebensgemeinschaften wurden kultivierungsunabhängig durch PCR und Denaturierende-Gradienten-Gel-Elektrophorese (DGGE) analysiert. Dafür wurden DNA-Primer verwendet, die auf einer universellen Region der 16S rRNA Gene, sowie auf zwei spezifischeren Regionen methylotropher α- und γ-*Proteobacteria* beruhen. Zwei weitere DNA-Primer amplifizierten das Gen der partikulären Methan-Monooxygenase (pMMO) *pmo*A und der Methanol-Dehydrogenase (MDH) *mxa*F. Die Kombination von DGGE-Analysen, Phospholipid (PLFA)-Analysen (Durchführung P. Roslev, Aalborg, DK) und Messungen des CH_4-Oxidationsprozesses, ermöglichten Aussagen über die Struktur, Funktion und Aktivität der methanotrophen Lebensgemeinschaft in den verschiedenen Habitaten.

Die methanotrophe Lebensgemeinschaft im Reisfeldboden bestand aus Populationen, die am nächsten zu den Typ I MOB *Methylobacter sp.* und *Methylococcus sp.* und den Typ II MOB *Methylosinus sp.* und *Methylocystis sp.* verwandt waren. Die Struktur dieser Lebensgemeinschaft zeigte eine hohe Dynamik, so nach Drainage und Düngung, als auch nach Inkubation des Bodens unter verschiedenen Bedingungen (CH_4, O_2, Feuchtigkeit). Typ II MOB waren im Reisfeldboden i. d. R. abundanter als Typ I MOB, insbesondere bei hohen CH_4-Mischungsverhältnissen. Unter niedrigen CH_4-Mischungsverhältnissen dagegen zeigten Typ I MOB eine sehr hohe Aktivität und bildeten auch mehr Biomasse als Typ II MOB. In bepflanzten Reismikrokosmen stimulierte NH_4^+-Zugabe Typ I MOB relativ stärker als Typ II MOB. Unter anoxischen Bedingungen, die eine CH_4-Oxidation nicht zuließen, waren Typ I MOB kaum oder nicht nachweisbar, wohingegen eine hohe Diversität innerhalb der Lebensgemeinschaft der Typ II MOB detektiert wurde, die offensichtlich unter diesen Bedingungen besser überleben konnten. Bereits bei geringer O_2 und CH_4 Verfügbarkeit bildete sich jedoch innerhalb weniger Tage eine sehr aktive, je nach Umweltbedingungen verschieden ausgeprägte Lebensgemeinschaft an Typ I MOB aus. Wohingegen die Lebensgemeinschaft der Typ II MOB auch unter veränderten Umweltbedingungen weitgehend konstant blieb.

Im Gegensatz zum Reisfeldboden wurde im sauren Waldboden das CH_4 aus der Atmosphäre oxidiert. Methanotrophe Populationen wurden über das *pmo*A-Gen nur in Bodenschichten detektiert, in denen auch atmosphärisches CH_4 oxidiert wurde. Die *pmo*A-Sequenzen waren nur entfernt mit den *pmo*A-Sequenzen bekannter Typ II MOB verwandt. Die detektierten Populationen stellen wahrscheinlich eine neue Gruppe methanotropher Bakterien dar, möglicherweise mit der Fähigkeit zur Oxidation von atmosphärischem CH_4. Dieses Ergebnis zeigte zusätzlich, dass der methodische Ansatz die Detektion unbekannter MOB ermöglichte.

Die entwickelte PCR-DGGE Methodik wurde weiterhin erfolgreich zur Kontrolle der Anreicherung und anschließenden Identifikation eines hoch-affinen Typ II MOB, Stamm LR1, aus einem Brachland angewandt (Dunfield *et al*., 1999).

II. Allgemeine Einleitung

Eine wesentliche Voraussetzung für Leben auf der Erde ist die Atmosphäre, deren Zusammensetzung durch Prozesse der Biosphäre beeinflusst wird.

Als Beispiel sei der Wechsel von der anoxischen zur oxischen Atmosphäre genannt, der durch die Entstehung der oxygenen Photosynthese durch Cyanobakterien verursacht wurde. Bis auf die Ausnahme der Edelgase (Ar, Ne, Kr und Xe), die sich langsam in der Atmosphäre anreichern, befinden sich fast alle Gase in einem Kreislauf mit der Biosphäre (Conrad, 1996). Lediglich die Kreisläufe der Spurengase Ozon und Radon unterliegen ausschließlich physikochemikalischen Prozessen (Conrad, 1995 b).

Im Wesentlichen verursachen die Gase CO_2, CH_4, N_2O und H_2O einen natürlichen "Treibhauseffekt". Ohne diesen natürlichen Treibhauseffekt läge die durchschnittliche Temperatur der Erdoberfläche bei ca. -18 °C.

Etwa 70 % der kurzwelligen Sonnenstrahlung (8,123 J cm^{-2} min^{-1} am Atmosphärenrand) erreicht in den mittleren Breiten den Meeresspiegel. Die von der Erde aufgenommene kurzwellige Energie wird als langwellige Strahlung (Infrarot, Wärme) wieder abgestrahlt, von den Treibhausgasen absorbiert und teils wieder zur Erde als Wärme abgestrahlt. Der Treibhauseffekt funktioniert analog der Aufwärmung in einem Gewächshaus (⇔Treibhaus), wobei die Treibhausgase die Funktion des Glasdachs übernehmen.

Etwa mit der Industrialisierung und der exponentiellen Bevölkerungszunahme ist ein kontinuierlicher Anstieg fast aller Treibhausgase zu verzeichnen. Der Anstieg der Treibhausgase (CO_2, CH_4, und N_2O) trägt wesentlich zur Erderwärmung bei, die als die größte der drohenden Gefahren für die mittelfristige Zukunft betrachtet werden kann.

1. Die Rolle des Methans in der Atmosphäre

Methan ist eines der wichtigsten Treibhausgase der Atmosphäre (Conrad, 1995 a & b; Dickinson & Cicerone, 1986; Ramanathan *et al*., 1987). Methan selber ist ein etwa 30-fach effektiveres Treibhausgas als CO_2 und daher trotz seines geringen Mischungsverhältnisses (derzeit 1,8 ppmv) von hoher Bedeutung (Conrad, 1997). Es fördert indirekt die Entstehung von Ozon in der Troposphäre und von Wasserdampf in der Stratosphäre, sowie den Abbau von Chlor-Radikalen die an der Ozonzerstörung in der Stratosphäre beteiligt sind (Crutzen, 1979; Crutzen & Schmailzl, 1983; Cicerone & Ormeland, 1988). Untersuchungen an Eisbohrkernen gaben Aufschluss über den Verlauf des atmosphärischen CH_4 Mischungsverhältnisses der letzten 160.000 Jahre (Chappellaz *et al*., 1990). Dabei hat sich gezeigt, dass sich allein in den vergangenen 100 Jahren das atmosphärische CH_4 Mischungsverhältnis

mehr als verdoppelt hat. Der jährliche CH_4-Anstieg von 0,8 % bis 1,0 % a^{-1} hat sich in den 90`er Jahren verlangsamt (Rudolph, 1994; Conrad, 1997).

2. Die Quellen des atmosphärischen Methans

Etwa 70-90 % des atmosphärischen CH_4 ist biogenen Ursprungs (Cicerone & Oremland, 1988; Heyer, 1990).

Da Methan durch strikt anaerobe methanogene Archaea gebildet wird, ist seine Bildung an anoxische Standorte gebunden. Wesentliche CH_4-Quellen sind Feuchtgebiete, also Sümpfe, Reisfelder, sowie Müll- und Klärschlammdeponien, und die anaeroben Verdauungstrakte von Wiederkäuern (Rinderhaltung) und Termiten (Conrad, 1997).

Nichtbiogenes CH_4 entsteht in geologischen Prozessen und bei der Verbrennung von Biomasse. Es entweicht bei der Erdöl-, Erdgasförderung, sowie durch defekte Pipelines in die Atmosphäre (Crutzen, 1991). Obwohl atmosphärisches CH_4 fast ausschließlich biogenen Ursprungs ist, ist die Quellengröße und die Dimension der Freisetzung stark an anthropogene Aktivität und Ausbreitung gekoppelt.

Wassergesättigte Böden (wetland soils), in denen aufgrund des hohen Wassergehaltes eine limitierte Sauerstoffdiffusion und dadurch anoxische Bedingungen vorherrschen, stellen mit 35 % den größten Anteil an der Methanemission (Cicerone & Oremland, 1988; Conrad 1995 a & b).

Das Hauptnahrungsmittel für 40-50 % der Weltbevölkerung ist Reis (Lindau *et al*., 1990). Etwa 80 % der Reisernte wird im ertragreicheren Nassanbau (wetland rice) produziert. Dabei werden die Reisfelder 4 Wochen vor der Pflanzung oder der Saat geflutet und erst kurz vor der Ernte wieder drainiert. Durch den Reisanbau sind riesige anthropogene Feuchtgebiete entstanden, die etwa 60 Tg a^{-1} CH_4 freisetzten und ca. 12 % des globalen CH_4 Budgets ausmachen (Prinn, 1994; Conrad, 1997; Milich, 1999).

Durch den wachsenden Bevölkerungsdruck wird bis zum Jahre 2020 eine Produktionssteigerung um 65 % von derzeit 460 Mt a^{-1} auf 760 Mt a^{-1} Reis prognostiziert (Neue, 1997). Dieser Bedarf kann nur durch eine Intensivierung des Anbaus erreicht werden (IRRI, 1989). Die Bedrohung durch „global warming“ und der Anstieg an Treibhausgasen macht das Verständnis für die in diesen Böden ablaufenden Prozesse unerlässlich. Nur dadurch können Strategien und adäquate Maßnahmen entwickelt werden, die zu einer Verminderung der CH_4-Freisetzung führen (Neue, 1997).

3. Die Senken des atmosphärischen Methans

Die größte Senke für CH_4 sind photochemische Reaktionen mit Hydroxyl (OH)-Radikalen in der Tropo- und Stratosphäre. In der Troposphäre werden auf diesem Weg 80-

85 % und in der Stratosphäre 10-15 % des atmosphärischen CH_4 abgebaut (Cicerone & Ormeland 1988, Crutzen, 1991). Neben dieser abiotischen CH_4-Senke stellt die CH_4-Oxidation durch MOB die einzige weitere Senke dar. In oxischen Böden werden 5 %-10 % oder bis zu 70 Tg a^{-1} CH_4 oxidiert (Conrad, 1995 a & b; King, 1997; Neue, 1997).

Die allgemein steigenden Spurengaskonzentrationen in der Atmosphäre führen zu einem vermehrten Abbau der OH-Radikale und vermindern die Kapazität dieser sehr wichtigen Senke einerseits für CH_4, andererseits für andere wichtige Reaktionen, wie die Zerstörung von Kohlenwasserstoffen, NO_x und SO_x (King, L.S. *et al*., 1989; Schimel & Gulledge, 1998). Damit übernimmt die biogene CH_4-Oxidation in Böden eine immer wichtigere Rolle. Selbst kleine Veränderungen in den CH_4-oxidierenden mikrobiologischen Prozessen können zu signifikanten Veränderungen des atmosphärischen CH_4-Mischungsverhältnisses beitragen (King, 1992; Ojima *et al*., 1993).

Der CH_4-Flux zwischen Biosphäre und Atmosphäre beruht also wesentlich auf den zwei mikrobiologischen Prozessen der Methanogenese und der Methanotrophie (Conrad, 1997).

MOB sind ubiquitär verbreitet und ein sehr wichtiger Bestandteil bei der Regulation der CH_4-Freisetzung. Der Anteil der atmosphärischen CH_4-Senke von 10 % spiegelt nur einen geringen Teil der wirklichen Regulationsleistung der Methanotrophen. In Reisfeldern und Sedimenten wird bis zu 97 % des gebildeten CH_4 nicht freigesetzt. Es wird von MOB in der oxischen, wenige Millimeter tiefreichenden Oberflächenschicht und an der oxischen Rhizosphäre von Reis und anderer hydromorpher Pflanzen noch vor der Emission in die Atmosphäre zu CO_2 und H_2O oxidiert (Conrad & Rothfuss, 1991; Frenzel *et al*., 1992; Gilbert & Frenzel, 1995; Calhoun & King, 1997, King, 1996; Van der Nat & Middleburgh, 1998).

Obwohl die MOB bei der CH_4-Oxidation CO_2 freisetzen, reduzieren sie effektiv die Treibhausgasmenge, da CH_4 einen 30-fach höheren Treibhausgaseffekt als CO_2 zeigt (Dickinson & Cicerone, 1986). Zudem wird ein Teil des oxidierten CH_4 in Biomasse inkorporiert (Manicelli, 1995).

Überwiegend in marinen Sedimenten wurde auch eine anaerobe CH_4-Oxidation mit SO_4^{2+} als Elektronenakkzeptor nachgewiesen, jedoch sind die darin involvierten anaeroben Methanoxidierer oder mikrobiellen Konsortien noch unbekannt (Mancinelli, 1995; Hoehler & Alperin, 1996; Harder, 1997; Hansen *et al*., 1998).

4. Methanotrophe Bakterien

Söhngen isolierte 1906 *Bacillus methanicus* (heute: *Methylomonas methanica*) das CH_4 als alleinige Kohlenstoff und Energiequelle nutzt (Söhngen,1906). Seit diesem ersten

isolierten methanotrophen Bakterium wurden seit etwa 1970 verstärkt weitere MOB isoliert und beschrieben (Whittenbury *et al*.,1970).

Die Methanotrophen bilden eine Untergruppe der ”methylotrophen Bakterien”. Methylotrophe Bakterien sind aerobe Mikroorganismen, die reduzierte C_1-Körper zu oxidieren vermögen. Es können aber nur MOB CH_4 als Kohlenstoff und Energiequelle nutzen.

Neben Bakterien wurde die Fähigkeit zur CH_4-Oxidation auch bei zwei Hefen, *Rhodotorula glutinis* und *Sporobolomyces roseus* beschrieben, was später aber nicht mehr verifiziert wurde (Wolf & Hanson, 1980).

Traditionell werden die MOB nach Art der C-Fixierung, Anordnung der intracytoplasmatischen Membranen, ihrer Morphologie und G+C-Gehaltes in die Typen I, II und X unterschieden (King, 1992; Mancinelli, 1995; Hanson & Hanson, 1996).

MOB assimilieren Kohlenstoff direkt aus der CH_4-Oxidation über das Zwischenprodukt Formaldehyd (CH_2O): Typ I MOB benutzen dazu den Ribulose-Monophosphat-Weg, Typ II MOB den Serin-Weg und Typ X MOB verfügen über beide Wege.

Nach 16S rDNA und 5S rDNA Analysen werden die Typ I MOB und Typ X MOB in die γ-Gruppe der Proteobakterien und die Typ II MOB in die α-Gruppe der Proteobakterien eingegliedert (King, 1992; Mancinelli, 1995).

Im Weiteren wird nur die Bezeichnung Typ I und Typ II MOB verwendet, dabei wird der Typ X aufgrund der 16S rDNA Phylogenie dem Typ I zugeordnet.

Bisher waren alle isolierten Stämme Gram-negative, stäbchen-, kokken-, oder vibrioförmige Zellen (Hanson & Hanson, 1996). Die Mehrzahl der Arten besitzt die Fähigkeit trocken- oder hitzebeständige Überdauerungsformen zu bilden. Methanotrophe sind überwiegend durch ein extensives, intrazelluläres Membransystem und einen sehr ähnlichen dissimilatorischen Stoffwechselweg gekennzeichnet (Mancinelli, 1995; Hanson & Hanson, 1996).

Der erste Oxidationsschritt des Methans zum Methanol wird durch die Methan-Monooxygenase katalysiert. Zwei Formen des Enzyms wurden charakterisiert, die lösliche Methan-Monooxygenase (sMMO) und die membrangebundene, partikuläre Methan-Monooxygenase (pMMO). Während alle bisher bekannten MOB (Ausnahme *Methylocella palustris*, s.u.) die pMMO besitzen, ist die sMMO nur in den MOB des Typs II und X, und bei *Methylomonas sp*. vorhanden. Die sMMO benutzt NADH als e^--Donator und enthält keine Häm- oder andere Kofaktoren. Die pMMO besteht aus drei Untereinheiten, enthält 2-3 Cu-Cluster im aktiven Zentrum und ist direkt an die Elektronentransportkette gekoppelt (Zahn & Dispirito, 1996; Nguyen *et al*., 1998).

Kürzlich wurde jedoch ein neues methanoxidierendes, acidophiles Bakterium, *Methylocella palustris,* beschrieben (Dedysh *et al*., 2000). Dieser neue methanoxidierende Stamm ist

ein α-*Proteobakterium*. Es ist aber nur entfernt verwandt mit anderen Typ II MOB. Es besitzt keine internen Membranstapel und bisher konnte nur die sMMO nachgewiesen werden.

Methanotrophe sind durch die Fähigkeit zur Methanoxidation gekennzeichnet, aber phylogenetisch und morphologisch unterschiedlich. Selbst phylogenetisch nahe verwandte Arten, wie z. B. verschiedene *Methylomonas* Arten besitzen unterschiedlich ausgeprägte Enzymsysteme, auch von nicht direkt an der CH_4-Oxidation beteiligten Enzymen. So enthält *Methylomonas sp*. Stamm 761 einen vollständigen Tricarbonsäurezyclus und wächst für mehrere Generationen auf Glucose als Substrat ohne seine spezifische CH_4-Oxidationsrate zu verlieren (zitiert nach: Hanson & Hanson, 1996).

5. Die hoch- und niedrig-affine CH_4-Oxidation

Wie bereits erwähnt kommen MOB in sehr unterschiedlichen Habitaten vor. Zum einen an den oxisch-anoxischen Grenzflächen methanogener Standorte (Reisfelder, Feuchtgebiete, Sedimente, Mülldeponien, u.a.), die durch hohe CH_4 Konzentrationen gekennzeichnet sind, zum anderen in oxischen Böden (z. B. Wald-, Ackerböden, Grünland, u.a.), in denen i.d.R. CH_4 nur in atmosphärischen Mischungsverhältnissen (1,8 ppmv) vorliegt. Die bestimmten kinetischen Parameter und die apparente halbmaximale Substrataffinität ($K_{m(app)}$) dieser Standorte unterscheidet sich um mehrere Größenordnungen (Conrad, 1995 a). Der $K_{m(app)}$ für CH_4 an methanogenen Standorten liegt im mikromolaren Bereich (niedrig-affin), während bei der Oxidation atmosphärischem Methans der $K_{m(app)}$ im nanomolaren Bereich (hoch-affin) liegt (Bender & Conrad, 1992, 1993; Bosse *et al*., 1993; Henckel & Conrad, 1998). Berechnungen auf Grundlage der kinetischen Parameter von Reinkulturen und Versuche mit *Methylosinus trichosporium* und *Methylobacter albus (*Synonym: *Methylomicrobium album)* ergaben, dass die isolierten MOB in Reinkultur nicht in der Lage sind, längere Zeit unter atmosphärischen CH_4-Konzentrationen zu überleben (Conrad, 1984; Roslev & King, 1994; Schnell & King, 1995; Benstead & King, 1997). Bender & Conrad (1992) prägten für dieses Phänomen den Begriff der bekannten niedrig-affinen MOB (common methanotrophs) und der unbekannten hoch-affinen MOB (unknown methanotrophs).

6. "Molekulare Fingerabdrücke" mikrobieller Lebensgemeinschaften

Obwohl die Prozesse der CH_4-Oxidation in Böden, Sedimenten, aquatischen und marinen Lebensräumen gut untersucht sind, ist die eigentliche methanotrophe Vergesellschaftung und Populationsstruktur in Böden weitestgehend unbekannt. Mit der Entwicklung der Molekularbiologie wurden neue kultivierungsunabhängige Methoden zur Untersuchung mikrobieller Populationsstrukturen geschaffen, durch die es ermöglicht wird, Einblicke in mikrobielle Lebensgemeinschaften im jeweiligen Habitat zu bekommen. Die dadurch, vor-

wiegend auf 16S rDNA Basis entstehende ”molekulare Diversität” macht jedoch letztendlich die wirkliche Isolierung der einzelnen Mitglieder der mikrobiellen Gemeinschaften nicht überflüssig (Liesack *et al.*, 1997).

Vermutlich sind bisher weniger als 1 % der bakteriellen Spezies isoliert (Amann, 1995). DNA-Reassoziationsexperimente deuteten auf bis zu 4000 verschiedene "Reassoziationstypen" in einem Gramm Boden hin, was bis zu 13000 verschiedene Spezies repräsentieren könnte (Torsvik *et al.*, 1996).

Als adäquat zur Untersuchung mikrobieller Lebensgemeinschaften und deren Änderungen haben sich die sogenannten ”Molekularen-Fingerprinting-Methoden” erwiesen. Fingerprinting-Methoden nutzen Zellbestandteile, also Moleküle oder Molekülgruppen, die bei allen zu betrachtenden Organismen vorhanden sind und auf Grund geringfügiger Unterschiede der Molekülstruktur oder Zusammensetzung eine Differenzierung einzelner Organismen oder Organismengruppen ermöglichen. Der überwiegende Teil der Methoden benutzt Nukleinsäuren, im Wesentlichen die 16S rDNA als Zielmolekül, aber auch Phospholipide können als molekulare Marker verwendet werden (Liesack *et al*., 1997; Heuer *et al*., 1997; Tiedje *et al*.,1999; Zelles, 1999).

Wie der Name bereits impliziert, liefern die Methoden einen ”Fingerabdruck” des jeweiligen Habitats. Dieser stellt in der Regel nicht die vollständige Diversität der mikrobiellen Populationsstruktur des Habitats dar, sondern lediglich einen methodisch und habitatspezifischen Teilaspekt.

6.1. Die Denaturierende-Gradienten-Gelelektrophorese

Die Denaturierende-Gradienten-Gelelektrophorese (DGGE) oder die Temperatur-Gradienten-Gelelektrophorese (TGGE) ermöglicht die Trennung gleich langer DNA-Moleküle. So werden z.B. PCR-Produkte während der Elektrophorese in einem Polyacrylamidgel mit denaturierenden Harnstoff-Formamid-Gradienten (DGGE) oder durch einen Temperaturgradienten (TGGE) auf Grund ihres sequenzspezifischen Schmelzverhaltens getrennt.

Ursprünglich wurde die DGGE zur Detektion von Punktmutationen in der medizinischen Forschung entwickelt (Fischer & Lermann, 1979). PCR-Fragmente mit bis zu einem Basenunterschied können in der DGGE differenziert werden (Muyzer *et al*., 1993; Heuer *et al*., 1997; Muyzer & Smalla, 1998). Muyzer *et al*. (1993) etablierte die DGGE für die Anwendung in der molekularen Ökologie zur Untersuchung mikrobieller Populationsstrukturen.

Ein Vorteil der DGGE gegenüber anderen Fingerprinting-Methoden, ist die Möglichkeit, die aufgetrennten Banden aus dem Gel zu extrahieren und zu sequenzieren (Ferris & Ward, 1997; Kowalchuk *et al*., 1997; Henckel *et al*., 1999; Dunfield *et al*., 1999). Damit bietet

DGGE einen direkten Zugriff auf phylogenetische Informationen über die jeweilige Populationsstruktur.

Eine weitere Möglichkeit, die Banden bestimmten Organismen zuzuordnen, ist die Hybridisierung der DGGE-Gele mit spezifischen Oligonukleotidsonden, wodurch bereits bekannte oder gesuchte Stämme in einem Habitat detektiert und nachgewiesen werden können (Brinkhoff & Muyzer, 1997; Santegoeds *et al*.,1998; Stephen *et al*., 1998; Kowalchuk *et al*., 1998; McCaig *et al*., 1999, Kowalchuk *et al*., 1999).

Durch die Auswahl spezifischer Primer können bestimmte Organismengruppen im Habitat untersucht werden. Gerade bei Organismen, wie den MOB, die physiologisch ähnlich aber phylogenetisch heterogen sind und somit auf 16S rDNA Basis nicht als eine Gruppe zu detektieren sind, bieten PCR-Systeme für Gene funktioneller, d. h. an Stoffwechselreaktionen beteiligter Enzyme, besondere Möglichkeiten.

6.2. Phospholipid-Muster

Ein wesentlicher Bestandteil der Membranen aller Organismen sind Phospholipide (Phospholipid Fatty Acids, PLFA). PLFAs sind auf die Membranen beschränkt, unterliegen schnellen Umsetzungsraten und kommen in relativ konstanten Konzentrationen pro Zelle vor (White, 1988). Anhand ihrer PLFA-Muster können Organismen über Zusammensetzung, Fettsäurelänge, und Lage der Doppelbindung in die verschiedenen Gruppen, bei Bakterien und Archaea in einzelnen Fällen sogar bis zur Art, zugeordnet werden (Findlay *et al*., 1989; Haack *et al*., 1994). Besonders MOB können, aufgrund ihrer ungewöhnlichen PLFA gut charakterisiert und teilweise bis zur Art differenziert werden (Nichols *et al*.,1987; Bowman *et al*., 1993). So enthalten Typ I MOB hauptsächlich die ungewöhnlichen einfach-ungesättigten 16:1 und 16:0 Fettsäuren, Typ II MOB enthalten hingegen fast ausschließlich 18:1 Fettsäuren (Bowmann *et al*., 1993).

PLFA-Muster ermöglichen nicht nur die Charakterisierung der Populationsstruktur, sondern ermöglichen auf Grund der relativ konstanten Konzentration pro Zelle eine Quantifizierung der Biomasse (Frostegard *et al.,* 1991; Sundh *et al*., 1995). Die schnellen Umsetzungsraten der PLFAs in der Zellmembran eröffnet die Möglichkeit zu Markierungsexperimenten mit radioaktiven oder stabilen Kohlenstoffisotopen, wodurch die aktiv am Stoffumsatz beteiligten Populationen detektiert werden (Boschker *et al*., 1998; Roslev *et al*., 1997; 1999). PLFA-Analyse kann so Informationen über tatsächliches Wachstum bestimmter Populationen im Habitat liefern (Tunlid & White, 1992; Vestal & White, 1989).

7. Ziele der Arbeit

Die Oxidation von CH_4 durch MOB ist ein wichtiger Prozess zur Regulation der CH_4-Freisetzung aus Reisfeldern. Die Verminderung dieser CH_4-Quelle ist von globaler Bedeutung.

Bei der CH_4-Freisetzung spielt die Bewirtschaftung der Reisfelder z. B. durch Düngung und Drainage eine wichtige Rolle. Prozessorientierte Studien hatten gezeigt, dass in wassergesättigten Böden die NH_4^+, CH_4, und O_2-Konzentrationen unmittelbar die CH_4-Oxidation regulieren (Schimel *et al*., 1993).

Oxische Böden sind die einzige terrestrische Senke für atmosphärisches CH_4 und damit für den globalen CH_4-Haushalt sehr bedeutend. Hier wird die CH_4-Oxidation proximal nur durch CH_4 und NH_4^+ reguliert, da O_2 im durchlüfteten Boden nicht limitierend ist (Schimel *et al*., 1993). Die atmosphärische CH_4-Aufnahme ist durch eine hohe Affinität gekennzeichnet und wird von wahrscheinlich unbekannten, methanoxidierenden Bakterien verursacht (Conrad, 1996).

Ausgangspunkt der Arbeit war die bis dahin unbekannte Populationsstruktur der methanotrophen Lebensgemeinschaft sowohl in anoxischen als auch in oxischen Böden. Hauptziel war daher die methanotrophe Lebensgemeinschaft im Reisfeldboden und im Waldboden zu charakterisieren und deren Populationsstruktur aufzuklären.

Im Reisfeld sollte weiterhin untersucht werden durch welche Ereignisse die methanotrophe Lebensgemeinschaft verändert wird. Hat die O_2, CH_4 und NH_4^+-Verfügbarkeit, die im Feld durch Drainage, Düngung und die Reispflanzen verändert und beeinflusst wird, einen Einfluss auf die methanotrophe Lebensgemeinschaft?

Die Aufklärung der methanoxidierenden Lebensgemeinschaft im Waldboden war von besonderem Interesse, da sich die Kinetik der atmosphärischen CH_4-Aufnahme von Reinkulturen und wassergesättigten Böden wesentlich unterscheidet und bisher kein Organismus mit der Fähigkeit zur atmosphärischen CH_4-Oxidation isoliert worden war.

Für diese Fragestellungen mussten Methoden etabliert werden, mit denen die Diversität, Abundanz und Veränderungen der methanotrophen Lebensgemeinschaft detektiert und dargestellt werden konnte. Mit DNA-Primern für funktionelle, enzymatische Gene und für das Gen der 16S rRNA sollten relevante Zielsequenzen der MOB durch PCR amplifiziert und durch DGGE analysiert werden. Besonders unter dem Gesichtspunkt, dass auch bisher noch unbekannte MOB erfasst werden sollten, waren die DNA-Primer zur Amplifikation von Untereinheiten der methanotrophen Schlüsselenzyme pMMO und MDH, die in allen damals bekannten MOB vorhanden waren, sehr vielversprechend.
Zusätzlich sollte durch radioaktive Markierungsexperimente und PLFA-Analyse Aktivität und Biomassezuwachs der methanotrophen Populationen bestimmt werden.

Durch die Verbindung von molekularbiologischen Untersuchungen und prozessorientierten Messungen sollte versucht werden, die methanotrophe Populationsstruktur, Diversität und Veränderungen ihrer Aktivität im Reisfeld und Waldboden zu charakterisieren und zu verfolgen.

III. Material und Methoden

Im Folgenden sind die entwickelten oder etablierten Kernmethoden noch einmal gesondert und detailliert aufgeführt.

(Für die hier nicht aufgeführten, aber verwendeten oder allgemein gebräuchlichen Methoden sei auf die jeweiligen Methodenabschnitte der einzelnen Veröffentlichungen/Kapitel verwiesen.)

1. Chemikalien

Alle verwendeten Chemikalien besaßen, sofern nicht anders angegeben, den Reinheitsgrad ”zur Analyse” und wurden von den Firmen Fluka (Buchs, Schweiz), Merck (Darmstadt), und Sigma (St. Louis, USA) bezogen. Enzyme und andere molekularbiologische Reagenzien wurden von Amersham (Braunschweig) und Biozym (Hessisch-Oldendorf) bezogen. Oligonukleotide wurden von MWG-Biotech (Ebersberg) synthetisiert.

Die eingesetzten Gase lieferte Messer-Griesheim (Düsseldorf). Es wurden die folgenden Gase und Reinheitsgrade verwendet:

Stickstoff (N_2)	99,996 %
Sauerstoff (O_2)	für medizinische Zwecke
Synthetische Luft (synth. Luft)	20,5 % O_2 , 79,5 % N_2
Methan (CH_4)	99,995 %
Kohlendioxid (CO_2)	99,995 %

2. Die untersuchten Böden

Die Charakterisierung der methanotrophen Lebensgemeinschaft wurde an einem i.d.R. wassergesättigten und überwiegend anoxischen Boden (Reisfeldboden) und an einem oxischen Boden (Waldboden) durchgeführt

2.1. Reisfeldboden

Der Reisfeldboden stammte aus einem Reisanbaugebiet bei Vercelli, Italien. Der Boden wurde dort im Frühjahr, 1993 (Henckel *et al*., 1999) und im Frühjahr, 1999 (Henckel *et al*., in prep. a & b) entnommen, getrocknet und bis zum Versuchsbeginn in Kunststoffwannen gelagert (Material: Polyethylen; Kaiser & Kraft, Stuttgart). Boden aus dem Frühjahr 1993 war bereits zur Anzucht von Reis in Pflanzwannen benutzt worden (vgl. Henckel & Conrad, 1998). Der luftgetrocknete Boden wurde in einem Backenbrecher (Typ BB1,

Retsch, Haan) zerkleinert und mit einer Siebmaschine (Dietz, Motoren GmbH & CO KG, Dettingen) auf Korngröße ≤ 2 mm gesiebt.

2.2. Waldboden

Der Waldboden, eine Braunerde unter einem Laubmischwald (überwiegend *Fagus sylvatica* mit *Quercus robur*) wurde im Januar (Winter) und im Juli, 1999 (Sommer) auf den Lahnbergen bei Marburg (N 51° 00´; E 09° 50´) entnommen. Die gestochenen Bodenkerne wurden ohne weitere Lagerung untersucht. Im organischen Oberboden A_h (2-6 cm) lag der pH_{H2O} bei 3,8 und stieg im mineralischem Unterboden B_v (6-28 cm) auf pH 4,3. Als Ausgangsgestein (C) lag Buntsandstein an. Die Bodenart wurde als lehmiger Sand mit einer maximalen Wasserhaltekapazität (% WHK) von 46 % im A_h und 39 % im B_v Horizont bestimmt.

2.3. Bestimmung der maximalen Wasserhaltekapazität (mWK) des Bodens

Offene Plastikzylinder wurden unten mit Filterpapier und Gaze geschlossen und etwa ¾ mit Boden gefüllt. Anschließend wurden sie zur Wassersättigung 1 h bis zur Bodenfüllhöhe in Wasser gestellt. Dabei musste darauf geachtet werden, dass kein Wasser von oben in den Zylinder lief. Anschließend wurden die Zylinder auf feuchtem Sand für 3 h equilibriert. Dann wurden etwa 10 g des wassergesättigten Bodens auf Uhrgläsern bekannten Gewichts eingewogen und bis zur Gewichtskonstanz bei 105 °C getrocknet, später im Exsikkator abgekühlt und gewogen (Schlichting & Blume, 1966).

$$mWK\ [\%\ H_2O\ gTS^{-1}] = (Fg-Tg) / (Tg-Lg) * 100$$

mWK: maximale Wasserhaltekapazität im Verhältnis zum Trockengewicht [% H_2O gTS^{-1}]

Fg: Feuchtgewicht [g]

Tg: Trockengewicht (getrocknet 105 °C) [g]

Lg: Leergewicht des Gefäßes [g]

3. Versuchsaufbau zur Durchflussinkubation

Um konstante CH_4 und O_2 Mischungsverhältnisse während der Inkubation zu gewährleisten, wurde ein Aufbau entwickelt, der einen konstanten Gasfluss durch das Inkubationsgefäß mit Boden ermöglichte. Um die technische Durchführung zu vereinfachen, wurde ein geschlossenes System gebaut, durch das die eingestellte Atmosphäre zirkuliert wurde. Ein 27 l Gasreservoir (Plastigas-Beutel, Roth, Karlsruhe) wurde gasdicht über eine Membranpumpe (FM1101 F, Fuergut, Aichstetten) und einem Durchflussmesser (0,2-2,0 l min^{-1}, Fuergut, Aichstetten) mit einem Erlenmeyerdurchflusskolben (1,1 l, Ochs, Göttingen) verbunden. Etwa 70 gFG Reisfeldboden (Vercelli, Frühjahr 1999) wurde eingewogen, mit einem Kautschukseptum verschlossen und gasdicht an das Durchflusssystem angeschlossen. Ein Gas:Boden-Verhältnis von > 400:1 (v:v) und die stete Gaszirkulation gewährleiste-

ten, dass die Mischungsverhältnisse von CH_4 und O_2 im System und im inkubierten Boden über einen absehbaren Zeitraum hinreichend konstant blieben (siehe Tabelle 1, S. 60). Die eingestellten O_2 und CH_4 Gasmischungsverhältnisse wurden gaschromatographisch gemessen und ggf. durch Zugabe von CH_4 und O_2 korrigiert. Bei Inkubationen mit O_2 Mischungsverhältnissen <20 %, wurde der Boden und Durchflusskolben vorher mit Stickstoff gespült.

Um ein Austrocknen des Bodens während der Inkubation zu verhindern, wurden in das Gasreservoir 10 ml steriles Wasser eingespritzt.

3.1. Behandlung des Bodens

Der Boden (Vercelli, Frühjahr 1999) wurde luftgetrocknet und zu Versuchsbeginn mit AMS-Medium auf 20 % gravimetrischen Wassergehalt angefeuchtet. AMS-Medium wurde verwendet, um eine NH_4^+-Limitation im Boden zu verhindern.

3.1.1. Ammonium-Mineralsalz (AMS)-Medium

NH_4Cl	0,5 g l^{-1}
KH_2PO_4	0,5 g l^{-1}

pH 6,8; nach dem Autoklavieren Zugabe von:

Spurenelementlösung SL10a	2 ml l^{-1}
Stammlösung $MgSO_4$	1 ml l^{-1}
Stammlösung $CaCl_2$	1 ml l^{-1}

Spurenelementlösung SL10a nach Widdel (Green, 1992)

HCl	50 mM
$FeCl_2$ x 4 H_2O	5 mM
$ZnCl_2$	0,5 mM
$MnCl_2$ x 2 H_2O	0,5 mM
H_3BO_3	0,1 mM
$CoCl_2$ x 6 H_2O	0,5 mM
$CuCl_2$ x 2 H_2O	0,01 mM
$NiCl_2$ x 6 H_2O	0,1 mM
Na_2MoO_4 x 2 H_2O	0,15 mM

Das $FeCl_2$ wurde in der entsprechenden Menge 2 M HCl gelöst, alle weiteren Verbindungen zugegeben und auf das gewünschte Volumen aufgefüllt. Die Lösung wurde in fest geschlossenen Gefäßen autoklaviert.

Stammlösungen für AMS

$CaCl_2$ x 2 H_2O	0,75 g in 50 ml
$MgSO_4$ x 7 H_2O	5 g in 25 ml

3.2. Probenentnahme

Zu bestimmten Zeitpunkten wurden Bodenproben für die molekulare Analytik und für radioaktive Markierungsversuche entnommen. Dazu wurde der Gasfluss kurzzeitig unterbrochen und jeweils etwa 5 gFG Boden aus dem Durchflusskolben entnommen. Danach wurden der Durchflusskolben wieder gasdicht verschlossen. Je nach eingestellter Atmosphäre wurde mit N_2 (bei 1 % O_2) oder synth. Luft gespült, anschließend die ursprünglichen O_2 und CH_4 Mischungsverhältnisse im Durchflusskolben wieder eingestellt und die Gaszirkulation durch das ganze System wieder geöffnet.

4. DNA Extraktion aus Böden

Zur Extraktion von Gesamt-DNA aus Boden wurde ein Direkt-Lyse-Verfahren angewandt, bei dem die Mikroorganismen direkt im Bodenmaterial selbst aufgeschlossen werden. Zur Extraktion der DNA aus Reisfeld- und Waldboden erwies sich eine DNA Extraktionsmethode, die nach Moré *et al*. (1994) modifiziert wurde, als am besten geeignet.

Der zu extrahierende Boden (0,5 bis 0,8 gFG) wurde in 2-ml Schraubdeckelreaktionsgefässe überführt. Etwa 1g hitzesterilisierte (180 °C für 4h) und Nukleinsäurefreie-Zirconium/Silizium Kugeln (∅0,1 mm; Biospec Products Inc., Bartlesville, Ok, USA), 800 µl Na-Phosphat-Puffer (120 mM, pH 8,0), und 260 µl Natriumdodecylsulfat (SDS)-Lösung (10 % SDS; 0,5 M Na-Phosphat-Puffer pH 8,0; 0,1 M NaCl) wurden zum Boden gegeben und durch vortexen homogenisiert. Die Zelllyse wurde in einer Zellmühle (FP120 FastPrep, Savant Instruments Inc., Farmingdale, NY, USA) für 45 s bei einer maximalen Beschleunigung von 6,5 m sec^{-1} durchgeführt. Zur Sedimentation der Festbestandteile wurde das Homogenisat für 3 min zentrifugiert (12.000 x *g*), der Überstand abgenommen und in ein sauberes 2-ml Reaktionsgefäss überführt. Das aus Boden und Zirkoniumkugeln bestehende Pellet wurde ein zweitesmal nur mit 700 µl Na-Phosphat-Puffer (120 mM, pH 8,0) resuspendiert. Nach erneuter Zentrifugation wurde der Überstand der zweiten Extraktion mit dem ersten Überstand vereinigt. Proteine, Zellbestandteile und andere Verunreinigungen wurden durch einen Prezipitationschritt für 5 min auf Eis mit 0,4 Volumenanteilen 7.5 M Ammonium-Acetat Lösung aus dem Bodenrohextrakt gefällt. Nach Zentrifugation bei 4 °C für 3 min (12.000 $\times$ *g*) wurde der Überstand vorsichtig abgehoben. Die DNA wurde mit 0,7 Volumenanteilen Isopropanol und Zentrifugation für 45 min bei 4 °C und 12.000 $\times$ *g* gefällt. Abschliessend wurde das DNA Pellet mit 1 ml 70 %igem Ethanol bei 4 °C gewaschen und nach dem Trocknen in 150 µl Tris-EDTA Puffer (10 mM Tris, 1 mM EDTA, pH 8,0) aufgenommen.

4.1. Verunreinigungen der Boden-DNA mit Huminsäuren

Aufgrund ähnlicher chemischer Eigenschaften werden Huminsäuren zusammen mit der DNA aus Boden extrahiert. Huminsäuren inhibieren die *in-vitro* DNA Synthese in der PCR. Aus Reisfeldboden extrahierte DNA enthielt relativ geringe Anteile an Huminsäuren. Eine Amplifikation durch PCR war nach einer Standardreinigung mit Glasmilch (Prep-a-Gene DNA Purification-Kit, Bio-Rad, München) und Verdünnung der DNA (1:100 in Wasser) meist möglich. Waldboden enthält jedoch so hohe Huminstoffkonzentrationen, dass die Boden- DNA als schwarzes Pellet extrahiert wurde. DNA Amplifikation war in keinem Fall möglich. Verschiedene DNA Extraktions- und Reinigungsmethoden wurden getestet. Die Reinigung der DNA durch Absorption der Huminstoffe an Polyvinylpolypyrrolidon (PVPP) erwies sich als optimal (Holben *et al*., 1988).

4.1.2. Reinigung der DNA von Huminsäuren

15 g PVPP (Sigma, St. Louis, USA) wurden in 200 ml 3 M HCl für 12-16 h durch Rühren suspendiert. Danach wurde das PVPP in 10 mM Tris-EDTA-Puffer pH 8,0 (TE-Puffer) equilibriert (Holben *et al*., 1988). Dazu lässt man das PVPP sedimentieren, ersetzt den Überstand mit TE-Puffer und rührt die Suspension einige Stunden. Dieser Vorgang wird mindestens zweimal wiederholt. Falls nötig, wird die PVPP-TE-Suspension mit NaOH auf einen pH 8,0 eingestellt. Die Suspension kann bei 4 °C gelagert werden.

Die PVPP-TE Suspension wurde in Mikro Bio-Spin Chromatographie Röhrchen (Bio-Rad, München) gefüllt und durch Zentrifugation 375 x *g*, 1 min direkt vor der Zugabe der DNA Lösung blasenfrei gepackt und trocken-zentrifugiert. 100-150 µl der in TE-Puffer gelösten DNA wurden auf die Säulenmatrix pipettiert und bei 375 x *g* für 2 min durch die PVPP-Matrix zentrifugiert. Da die Absorptionskapazität der PVPP für Huminstoffe begrenzt ist, muss bei extrem hohen Huminstoffkonzentrationen das DNA-Eluat ein weiteres Mal über eine neue Säule gereinigt werden, oder ein geringeres DNA-Volumen aufgetragen werden.

Die eluierte DNA Lösung war klar und konnte durch eine PCR amplifiziert werden.

4.2. Konzentrationsbestimmung von DNA-Lösungen

Die Konzentration von DNA Lösungen wurde durch Bestimmung der Absorption (A_{260}) einer 1:10 Verdünnung in Wasser bei 260 nm in einem Photometer (Gene Quant, Pharmacia, Upsala, Schweden) bestimmt. Wobei die Konzentration doppelsträngiger DNA nach,

C [ng μl^{-1}] = 50 x Verdünnungsfaktor x A_{260} (Sambrook *et al*., 1989)

berechnet wurde. Dabei wird vorausgesetzt, dass die optische Dichte von 1 in 1 ml Wasser einer DNA-Menge von 50 µg entspricht.

5. Die Molekularen Analysen

Zur Charakterisierung der methanotrophen Lebensgemeinschaften wurde die kultivierungsunabhängige “Fingerprinting”-Methode DGGE verwendet. Die DGGE ermöglicht es kurze DNA-Moleküle (bis ca. 600 bp) gleicher Länge (PCR-Produkte) aufgrund unterschiedlichem Schmelzverhaltens durch geringfügige Sequenzunterschiede in einem denaturierenden Gradienten zu trennen (Fischer & Lerman, 1983; Muyzer *et al*., 1993). Um ein vollständiges Denaturieren der Doppelstrang DNA (dsDNA) in Einzelstränge (ssDNA) zu verhindern, wird an die DNA Fragmente während der PCR eine ca. 40 bp lange GC-reiche Region (GC-Klammer) synthetisiert. Dazu muss an das 5´-Ende eines der beiden Oligonukleotid-Primer eine GC-Klammer angehängt werden.

Es wurden 5 PCR-DGGE Systeme erfolgreich entwickelt und angewendet, die auf verschiedenen, bereits veröffentlichten Oligonukleotid-Primern beruhten (Tabelle 1). Es wurden drei Oligonukleotid-Primerpaare verwendet, die Abschnitte des 16S rRNA Gens amplifizierten und 2 Oligonukleotid-Primerpaare, die “funktionelle” Gene der pMMO und MDH amplifizierten. Die 16S rDNA Oligonukleotid-Primerpaare banden an Regionen i) hochkonserviert in allen Organismen (Universal), ii) spezifisch für methylotrophe α-Proteobakterien (MB9α), und iii) spezifisch für methylotrophe γ-Proteobakterien (MB10γ) (Tabelle 2). Die funktionellen Oligonukleotid-Primerpaare amplifizierten jeweils die α-Untereinheiten der pMMO (*pmo*A), und der MDH (*mxa*F), beides Schlüsselenzyme in MOB (Tabelle 2).

Tabelle 1 Verwendete Oligonukleotid-Primer. Für die DGGE-Analyse wurden die markierten Primer mit der entsprechenden GC-Klammer in der PCR verwendet.

Primer-Bezeichnung	Sequenz 5´-3´	Quelle
M13 uni	CGACGTTGTAAAACGACGGCCAGT	Promega (Mannheim)
M13 r	CAGGAAACAGCTATGAC	Promega (Mannheim)
A1892	GGSGACTGGGACTTCTGG	Holmes et al., 1996
A682	GAASGCNGAGAAGAASGC	Holmes et al., 1996
9α f	GTTCGGAATAACTCAGGG	Tsien et al., 1990
10γ f	AAGCGGGGGATCTTCGGACC	Tsien et al., 1990
533 r1	TTACCGCGGCTGCTGGCAC	Weisburg et al., 1991
533 f	GTGCCAGCAGCCGCGGTAA	Weisburg *et al*., 1991
907 r[1]	AATTCCTTTGAGTTT	Weisburg *et al*.1991
mxaF f	GCGGCACCAACTGGGGCTGGT	McDonald *et al*., 1995
mxaF r[2]	GGGCAGCATGAAGGGCTCCC	McDonald *et al*., 1995
GC-Klammer 1	cgcccgccgcgccccgcgcccggcccgccgcccccgcccc	Henckel *et al*., 1999
GC-Klammer 2	ccccccccccccccgccccccgcccccgcccccgccgccc	Henckel *et al*., 1999

S=G/C; N=A/T/G/C

5.1. Entwicklung der GC-Oligonukleotid-Primerpaare

Bei der Entwicklung der GC-Oligonukleotid-Primerpaare wurde der geeignetere GC-Primer und eine sinnvolle GC-Sequenz (d.h. geringste Neigung zu Primer-Primer-Paarungen oder “Hairpin”-Bildung) über Computerprogramme (http://www.idtdna.com/html/analysis/calc.html) theoretisch ermittelt. Der hohe GC-Gehalt eines Primers, der große Längenunterschied und die daraus resultierenden sehr unterschiedlichen Annealingtemperaturen des jeweiligen GC-Oligonukleotid-Primerpaares erforderte i. d. R. eine intensive Optimierung der Reaktionsbedingungen und des Temperaturprofils in der PCR. Eine zügige Optimierung konnte durch Gradienten-PCRs auf einem speziellen Thermocycler (Mastercycler Gradient; Eppendorf, Hamburg) erreicht werden. Dadurch konnten während eines Amplifikationsansatzes 12 verschiedene Annealingtemperaturen über einen maximalen Temperaturgradienten von 10 °C gleichzeitig getestet werden.

5.2. Amplifikation der DNA mittels PCR

Die verwendeten Oligonukleotid-Primerpaare für die *in-vitro* DNA Synthese sind in Tabelle 2 aufgeführt. Die PCR-Ansätze wurden in einem Gesamtvolumen von 50 µl durchgeführt. Als Negativkontrolle wurde bei jeder Amplifikation ein Reaktionsansatz mit dem entsprechendem Volumen Wasser anstelle von Matrizen-DNA versehen. Das Reaktionsgemisch bestand aus Taq-PCR Puffer (20 mM Tris-HCl, pH 8.3, 50 mM KCl; PE Applied Biosystems, Weiterstadt), 1 U *AmpliTaq* DNA Polymerase (Perkin Elmer Applied Biosystems, Weiterstadt, Germany), 0.5 µM pro Primer, 100 µM pro Desoxynukleotidtriphosphat (Amersham Life Science, Braunschweig, Germany) und 1-5 ng der jeweiligen Matrizen-DNA mit sterilem Wasser (Braun, Melsungen) wurden die Ansätze auf das Endvolumen von 50 µl aufgefüllt. Bei den gekennzeichneten Oligonukleotid-Primerpaaren (Tabelle 2) wurde die PCR mit vorgefertigten PCR-Reaktionsgemischen durchgeführt, so dass nur 1-5 ng der jeweiligen Matrizen-DNA, 1 U *AmpliTaq* DNA Polymerase mit sterilem Wasser auf das Endvolumen von 50 µl aufgefüllt wurde. Der MasterAmp 2 $\times$ PCR Premix enthielt 100 mM Tris-HCl (pH 8.3), 100 mM KCl, 5 mM $MgCl_2$ (Premix E) oder 7 mM $MgCl_2$ (Premix F), 400 µM pro Desoxynukleotidtriphosphat, sowie einen 4-fach konzentrierten, nicht näher spezifizierten “PCR-Enhancer” (Epicentre Technologies, Madison, WI, USA). Alle PCR-Ansätze wurden in gekühlten Reaktionsgefäßen angesetzt und umgehend in den auf 94 °C vorgeheizten Thermocycler-Block gestellt. Zur Amplifikation wurde der Mastercycler Gradient (Eppendorf, Hamburg) oder das PCR System 9700 (Perkin Elmer Applied Biosystems, Weiterstadt) verwendet.

Für die Reamplifikation ausgeschnittener und eluierter DGGE-Banden wurden die PCR-Reaktionsgemische in einem Endvolumen von 100 µl angesetzt.

Tabelle 2 PCR und DGGE-Bedingungen für die Oligonukleotid-Primerpaare. Die verwendeten GC-Klammern wurden an den jeweilig markierten Primer synthetisiert.

Oligonukleotid-Primerpaar	Zielgen	Größe des Amplikons [Bp]	PCR -Bedingungen			DGGE Gradient Zeit [h] Spannung [V]
			Temperatur profil	Anzahl der Zyklen	Reaktions ansatz	
UNI 533f/907r*	16S rRNA	392	94°C, 30 s 60-50°C, 30 s 72°C, 45 s	30	1,5 mM MgCl	35-70 % 5 h 150V
MB9α 9α f / 533r*	16S rRNA	391	94°C, 3 min 94°C, 1 min 60°C, 30 s	31	Premix F	40-70 % 5 h 150 V
MB10γ 10γ f / 533r*	16S rRNA	336	94°C, 1 min 60°C, 30 s 72°C, 1 min	29	Premix F	40-70 % 5 h 150 V
pmoA A189**/A682	*pmo*A α-Untereinheit pMMO	525	94°C, 1 min 62-55°C, 30 s 72°C, 45s	30	Premix F	35-80 % 6 h 200V
mxaF f1001/ r1557**	*mxa*F α-Untereinheit MDH	550	94°C, 1 min 55°C, 30 s 72°C, 1 min	31	Premix E	35-70 % 5 h 150V

***cgcccgccgcgccccgcgcccggcccgccgcccccgcccc**
****ccccccccccccgcccccgcccccgcccccgcccccgccgccc**

5.3. Denaturierende-Gradienten-Gel-Elektrophorese (DGGE)

Die PCR-Produkte wurden mit dem Dcode System (Bio-Rad, München) in 1-mm dicken Polyacrylamidgelen (6,5 % w/v Acrylamid:Bis Acrylamid (37,5:1); Bio-Rad, München) in 0,5 x TAE-Puffer, pH 7,4 aufgetrennt.

10 x TAE Puffer, pH 7,4

Tris-HCl	0,8 M	48,5 g l^{-1}
Natriumacetat	0,4 M	21,8 g l^{-1}
EDTA	0,02 M	4,15 g l^{-1}

Der pH Wert 7,4 wird mit Essigsäure eingestellt.

Polyacrylamid Stammlösung 0 %-denaturierend

		0,5 l Lösung
Acrylamid: Bis-Lösung (37,5:1)	6,5 %	81 ml
10 x TAE, pH 7,4	0,5 X	25 ml
Demineralisiertes Wasser *ad* 500 ml		

Polyacrylamid Stammlösung 80 %-denaturierend

		0,5 l Lösung
Acrylamid: Bis-Lösung (37,5:1)	6,5 %	81 ml
10 x TAE, pH 7,4	0,5 X	25 ml
Harnstoff , Urea	5,6 M	168 g
Formamid, deionisiert	32 %	160 ml
Demineralisiertes Wasser *ad* 500 ml		

Es wurden jeweils 11 ml der benötigten Ausgangslösungen mit minimalem und maximalem denaturierendem Agenz durch Mischen der 0 % und 80 %igen-Polyacrylamid-Stammlösungen im jeweiligen Verhältnis hergestellt. Das Gradientengel wurde mit 7 ml der 0 %igen Polyacrylamidlösung abgeschlossen. Die Polymerisierungsreaktion wurde durch Zugabe von insgesamt 145 µl frisch angesetzter 10 %iger Ammonium-Persulfat-Lösung und 14,5 µl Temed (Bio-Rad, München) im Endvolumen von 29 ml Polyacrylamidlösung pro Gel gestartet.

Das Gradientengel wurde über einen Gradientenformer, der mit einer Pumpe verbundenen war, durch gleichmäßiges Befüllen der Gel-Apparatur mit den entsprechend konzentrierten Polyacrylamidlösungen erzeugt. Das denaturierende Gel wurde mit 7 ml der 0 %igen Polyacrylamidlösung, das als Sammelgel diente und den Taschenbildungskamm aufnahm, überschichtet. Die spätere Handhabung der Gele wurde durch die Verwendung von GelBond PAG Folien (FMC Bioproducts, Rockland, ME, USA), auf die das Gel in der Gelapparatur gegossen wurde, erleichtert. Die Gele wurden über Nacht bei Raumtemperatur polymerisiert.

Die Elektrophorese wurde bei konstanter Spannung und 60 °C durchgeführt (Tabelle 2). Vor dem Auftragen der DNA-Proben wurden die Gele für 45 min in der temperierten DGGE-Einheit im elektrischen Spannungsfeld eingespannt und “vorlaufen” gelassen. Nach der Elektrophorese wurden die Gele in einer 1:50.000 Verdünnung mit dem DNA interkalierendem Fluoreszenzfarbstoff Sybr-green I (Biozym, Hessisch-Oldendorf) für 30 min gefärbt und anschließend mit einem Phosphorimager (Molecular Dynamics, Sunnyvale, Ca, USA) aufgezeichnet, digital gespeichert und ausgewertet.

5.3.1. Optimierung der DGGE Bedingungen

Der denaturierende Harnstoff/Formamid Gradient für eine optimale Bandentrennung der PCR-Produkte eines jeweiligen Oligonukleotid-Primerpaare wurde durch “perpendicular” DGGE ermittelt (Muyzer *et al*., 1997). Dabei wird ein Gradientengel mit einem breit angelegtem Harnstoff/Formamid Konzentrationsunterschied (z.B. 0-100 %) gegossen und das zu trennende PCR-Produkt in einer einzigen großen Geltasche über die gesamte Breite des Gels aufgetragen. Der Gradient wird bei diesem Gel derart gegossen, dass er senkrecht (perpendicular) zur Laufrichtung ausgerichtet ist (im Gegensatz zu “normalen” Gelen bei denen der Gradient parallel, also mit der Laufrichtung ausgerichtet ist). Nach Elektrophorese und Färbung des Gels erhält man eine S-förmig verlaufende DNA-Bande, die dadurch entsteht, dass die DNA bei unzureichender Harnstoff/Formamid Konzentration nicht denaturiert und mit hoher elektrophoretischer Mobilität weit durch das Gel läuft. Im Bereich zu hoher Konzentration kommt es ebenfalls zu keiner Trennung, da die DNA sofort denaturiert und sehr geringe elektrophoretische Mobilität zeigt oder in Einzelstränge zer-

fällt. Der Bereich optimaler Harnstoff/Formamid Konzentration wird durch die Auffächerung des PCR-Produktes in einzelne DNA-Banden deutlich.

Die nötige Laufzeit der jeweiligen PCR-Produkte in der DGGE wurde in “time-travel” Experimenten bestimmt. Dabei werden gleiche PCR-Produkte während der Elektrophorese zeitlich versetzt in unterschiedlichen Geltaschen in einem parallelen Gradientengel (“normale” Gradientenausrichtung) aufgetragen.

Die verwendeten DGGE-Bedingungen für jedes Oligonukleotid-Primerpaar sind in Tabelle 2 aufgeführt.

5.3.2. Ausschneiden einzelner DNA-Banden aus DGGE-Gelen

Der DNA Farbstoff Sybr-Green I wird bei einer Wellenlänge von 497 nm angeregt und emittiert bei einer Wellenlänge von 520 nm (Molecular Probes, Eugene, Oreg. USA). Damit wird es möglich, DNA im nicht-UV-Bereich sichtbar zu machen und die zu sequenzierende oder klonierende DNA vor UV-induzierter Schädigungen zu schützen. Die mit Sybrgreen I gefärbten DNA-Banden der DGGE-Gele wurden bei einer Wellenlänge > 400 nm auf einem Durchlichtgerät (Dark Reader; Clare Chemical Research, Ross on Wye, UK) sichtbar gemacht.

Einzelne DNA-Banden wurden mit einer abgeschnittenen, sterilen 200-µl-Pipettenspitze ausgestochen und in 200 µl sterilem und DNA/RNA-freiem Wasser (Braun, Melsungen) eluiert.

Die DNA der ausgestochenen DGGE-Banden wurde in einer erneuten PCR (s.o.) amplifiziert und nochmals durch DGGE reanalysiert. Nach der Reamplifikation durfte nur noch die eine, ausgestochene Bande in der DGGE-Analyse sichtbar sein. Nur dann konnte die DNA dieser DGGE-Bande sequenziert werden.

5.3.3. Klonierung von MB9α DGGE-Banden

Direkt aus DGGE-Gelen ausgeschnittene und reamplifizierte MB9α Banden zeigten bei der erneuten DGGE-Analyse Mehrfachbanden. Damit war eine direkte Sequenzierung der reamplifizierten MB9α Banden nicht möglich. Die reamplifizierten PCR-Produkte der MB9α Banden wurden daher vor der Sequenzierung kloniert.

Bei Klonierung des MB9α PCR-Produkt der ursprünglichen Amplifikation aus Umwelt DNA wurde festgestellt, dass die durch Klonierung bestimmte Diversität der Populationsstruktur nicht vollständig mit der in der DGGE bestimmten Populationsstruktur übereinstimmte (Henckel *et al.*, 1999). Deshalb wurde nachfolgend für die Sequenzierung jeweils die DNA der eigentlichen MB9α DGGE-Banden kloniert.

Zur Klonierung wurde das pGEM-TEasy Cloning kit (Promega, Madsion, WI) laut Hersteller Angaben verwendet. Die reamplifizierte DNA, der ausgestochenen DGGE-Bande wurde in

den pGEM-TEasy Vektor ligiert und anschließend in die mitgelieferten kompetenten *E. coli* Zellen transformiert. Klone mit vollständigem MB9α Insert wurden durch Blau-Weiß-Selektion auf LB-AMP-IPTG-Agarplatten identifiziert (Rotthauwe *et al*., 1997). Das klonierte Insert zufällig ausgesuchter Klone (8 Klone pro Bande) wurde mit Vector spezifischen Primern (M13) amplifiziert. Die M13-PCR-Produkte wurden mit dem DNA Aufreinigungskit Easy-Pure (Biozym, Hessisch-Oldendorf) gereinigt.
Durch erneute DGGE-Analyse mussten die klonierten MB9α Fragmente mit den ursprünglich aus der Umwelt DNA amplifizierten MB9α Banden verglichen werden. Dazu wurde das gereinigte M13-PCR-Produkt als Matrize (0,25 µl) mit dem MB9α Oligonukleotid-Primerpaar amplifiziert. Die somit wiedererhaltenen MB9α Fragmente wurden dann im Vergleich mit den ursprünglichen MB9α Produkten der Umwelt per DGGE analysiert. Alle klonierten MB9α Fragmente lagen nun als Einzelbanden vor. Klone, deren MB9α Inserts mit der jeweiligen Umwelt-MB9α Bande übereinstimmten, wurden nachfolgend sequenziert.

5.3.4. Sequenzierung von DGGE-Banden

Die PCR-Produkte der reamplifizierten, ausgeschnitten DNA-Banden wurden mit dem EasyPure-DNA-Reinigungskit (Biozym, Hessisch-Oldendorf) gereinigt und konzentriert. Die Konzentration und Reinheit der amplifizierten DNA wurde durch Absorption einer 1:20 Verdünnung in Wasser bei 260 nm bestimmt (s. o.).

Die Sequenzierung wurde mit dem „ABI PRISM™ Dye Teminator Cycle Sequencing Ready Reaction Kit“ mit „AmpliTaq DNA Polymerase, FS“ (PE Applied Biosystems, Weiterstadt) auf einem GeneAmp PCR System 9600 (PE Applied Biosystems, Weiterstadt) nach Herstellerangaben durchgeführt. Die Sequenzierreaktionen wurde in einem Endvolumen von 20 µl mit ca. 100 ng Matrizen-DNA, 5 pmol des jeweiligen Sequenzierprimer und 6 µl des “Terminator Ready Reaction Mix” durchgeführt. Als Sequenzierprimer wurde jeweils der dem PCR-Produkt entsprechende Oligonukleotid-Primer ohne GC-Klammer benutzt (Tabelle 1). Das Temperaturprofil für die zyklische Sequenzierung bestand aus einer initialen Denaturierung bei 94 °C für 3 min, gefolgt von 25 Zyklen bestehend aus 96 °C, 10 sec; 50 °C, 5 sec; 60 °C, 4 min und schliesslich einer Kühlung auf 4 °C.

Die PCR-Produkte wurden dann von überschüssigen Primern und Dye-Terminatoren mit Microspin G-50 Chromatographiesäulen (Pharmacia, Upsala, Schweden) gereinigt. Das Eluat wurde unter Vakuum getrocknet und das DNA-Pellet in 2 µl Formamid/EDTA (25 mM, pH 8,0) 4:1 (v/v) aufgenommen, 3 min bei 100 °C denaturiert und auf einem 5 %igem Polyacrylamidgel mit 8 M Harnstoff aufgetragen. Die Elektrophorese und Detektion erfolgte in einem automatischen Sequenziergerät (373A DNA Sequencer, PE Applied Biosystems, Weiterstadt) für 14 h bei 1500 V.

Diese nicht-radioaktive, zyklische Sequenzierung arbeitet nach dem Prinzip der Kettenabbruch-Methode nach Sanger *et al*. (1977). Dabei bewirken die in einem Reaktionsansatz enthaltenen 4 unterschiedlich Fluoreszenz-markierten Desoxynukleotid-Diphosphate (Dye-Terminatoren) einen Abbruch der Polymeraseaktivität während der *in-vitro* DNA Synthese. Dadurch erhält man nach Abschluss der Sequenzierung statistisch DNA-Moleküle aller Fragmentlängen von 1 bis n Basen (n ist die Länge des zu sequenzierenden DNA-Moleküls) deren letzte Base Fluoreszenz-markiert ist. Dieses Gemisch unterschiedlich langer Fluoreszenz-markierter DNA-Fragmente wird dann auf einem Polyacrylamidgel in einem automatischen Sequenziergerät getrennt. Hier erfolgt eine Trennung der DNA-Fragmente nach Molekülgröße (Länge). Passieren die DNA-Fragmente die Detektionseinheit wird die Fluoreszenz durch einen Laser angeregt, die Emission durch einen Photomultiplier erfasst und computergestützt in eine Elektropherogramm umgesetzt, welches die Basensequenz der Ziel-DNA zeigt.

6. Phylogenetische Einordnung

Die phylogenetische Einordnung der 16S rDNA Sequenzen diente der Identifikation einzelner Populationen der dargestellten Lebensgemeinschaft im Boden. Bei den 16S rDNA Sequenzen handelte es sich um Partialsequenzen von bis zu 350 bp Länge (vollständige 16S rDNA ca. 1500 bp), mit denen eine phylogenetische Einordnung im Sinne der Darstellung und Aufklärung evolutiver Entwicklung und Ereignisse aufgrund der begrenzten Sequenzinformationen nicht zulässig ist (Ludwig *et al*., 1998).

6.1. Sequenzverarbeitung

Die Rohsequenzen wurden computergestützt mit dem Programm Seqman der Lasergene-Software (DNA-Star, Madison, Wis, USA) editiert. Beide DNA-Stränge wurden je von 5´-3´und von 3´-5´ sequenziert. Die jeweiligen “Vorwärts-” und “Rückwärtsstränge” wurden komplementär verglichen und eine sog. Konsensussequenz aus beiden Strängen erstellt. Die jeweiligen 5´ und 3´-ständigen Primersequenzen wurden eliminiert und nicht bei der phylogenetischen Verrechnung berücksichtigt

6.2. Phylogenetische Einordnung der partiellen 16S rRNA-Gensequenzen

Die 16S rDNA Sequenzen wurden zur ersten Identifikation über die Funktion “BLAST-Search” gegen die Datenbank des NCBI Genbank (http://www.ncbi.nlm.nih.gov/) verglichen. Das Programm ermittelt die nächstähnlichen Sequenzen zur Zielsequenz.

Die phylogenetische Verrechnung und die Schätzung der Dendrogramme erfolgte computergestützt mit der ARB-Software (Strunk & Ludwig, 1996). Die 16S rDNA Sequenzen wurden mit der ARB-Software gegen ca. 5000 16S/18S rDNA Sequenzen der Domänen *Bacteria*, *Archaea* und *Eucarya* angeordnet (Alignment), d.h. jeweils homologe Nukleotidpositionen des 16S rRNA Gens wurden in Spalten einander zugeordnet. Das automati-

sche Alignment wurde gegen die vom Programm ermittelten nächstähnlichen Sequenzen überprüft und ggf. korrigiert.

ARB ermöglicht es, Partialsequenzen in auf Vollsequenzen basierenden, verifizierten Stammbäumen einzurechnen, ohne die Baumtopologie durch fehlende Nucleotidinformationen zu verfälschen. Die Partialsequenzen wurden in bereits bestehende, verifizierte Stammbäume integriert, die auf den derzeit verfügbaren 16S rDNA Datenbanken beruhen. Diese Einordnung wurde nach “Maximum-Parsimony”-Kriterien vorgenommen, bei der die Zielsequenzen denjenigen Sequenzen zugeordnet wird, für die die geringste Zahl an Nukleotidaustauschen berechnet wurde. Es wurden jeweils verschiedene Bäume basierend auf Maximum-Parsimony und Distanzverfahren (“Neigbor-joining” Algorithmus) verglichen. Distanzverfahren beruhen auf dem paarweisen Sequenzvergleich der zu analysierenden Sequenzen, wobei multiple Nukleotidsubstitutionen auf einer Position basierend auf verschiedenen Evolutionsmodellen berücksichtigt werden. Die Distanzwerte wurden durch Algorithmen basierend auf den Evolutionsmodellen nach Jukes-Cantor und Felsenstein, die in ARB implementiert sind, in ein Dendrogramm umgerechnet und dargestellt (Jukes & Cantor, 1969; Felsenstein, 1993).

6.3. Phylogenetische Einordnung der funktionellen Gensequenzen *pmo*A und *mxa*F

Bei Proteinen und Enzymen liegt der evolutive Selektionsdruck auf der Aminosäuresequenz. Daher wurde die phylogenetische Distanz der funktionellen Gene *pmo*A und *mxa*F auf Basis der Aminosäuresequenz berechnet. Die Nukleotidsequenzen der Gene *pmo*A und *mxa*F wurden in die drei möglichen Leseraster der Aminosäuresequenzen translatiert und der richtige Leserahmen determiniert. Mit der ARB-Software wurden die sequenzierten Gene zusammen mit aus der NCBI Datenbank “Genbank” (http://www.ncbi.nlm.nih.gov/Entrez/) erhaltenen *pmo*A oder *mxa*F Sequenzen gegenseitig angeordnet. Die Phylogenien wurden basierend auf dem Distanzverfahren “Neigbor-Joining” erstellt. Zur Baumberechnung wurden jeweils alle verfügbaren *pmo*A oder *mxa*F Sequenzen herangezogen.

IV. Ergebnisse

1. Die Charakterisierung der methanotrophen Lebensgemeinschaft im Reisfeldboden, durch molekulare Analyse des Gens der 16S rRNA, der partikulären Methan-Monooxygenase und der Methanol-Dehydrogenase

Die Zusammensetzung der methanotrophen Lebensgemeinschaft im Reisfeldboden wurde kultivierungsunabhängig durch molekularökologische Methoden charakterisiert. Dazu wurden verschiedene PCR-DGGE-Systeme auf Basis von drei Zielregionen des 16S rRNA-Gens und den Genen *pmo*A, der partikulären Methan-Monooxygenase und *mxa*F, der Methanol-Dehydrogenase angewendet.

Die methanotrophe Lebensgemeinschaft im Reisfeldboden bestand aus Populationen, die am nächsten mit den Typ I MOB *Methylobacter sp*. und *Methylococcus sp*. und den Typ II MOB *Methylosinus sp*. und *Methylocystis sp*. verwandt waren.

Die Untersuchungen zeigten, dass die starke Erhöhung der CH_4-Oxidationsraten -die sog. Induktion der CH_4-Oxidation- mit einem Populationswachstum der methanotrophen Lebensgemeinschaft einherging.

Applied and Environmental Microbiology, May 1999, p. 1980-1990

Molecular analyses of the methane-oxidizing microbial community in rice field soil by targeting the genes of the 16S rRNA, particulate methane monooxygenase, and methanol dehydrogenase

Thilo Henckel, Michael Friedrich, Ralf Conrad

Abstract

Rice field soil at non-saturated water content induced CH_4 consumption activity when supplemented with 5% CH_4. After a lag phase of 3 days CH_4 was consumed rapidly to concentrations below 1.8 parts per million by volume (ppmv). However, the soil was not able to maintain the oxidation activity at near-atmospheric CH_4 mixing ratios (i.e. 5 ppmv). The soil microbial community, was monitored by denaturing gradient gel electrophoresis (DGGE) during the oxidation process using different PCR primer sets based on the 16S rRNA gene, and on functional genes. A universal SSU rDNA primer set, and 16S rDNA primer sets specifically targeting type I (γ-*Proteobacteria*) and type II (α-*Proteobacteria*) methanotrophs were used. Functional PCR primers targeted the genes of particulate methane monooxygenase (*pmoA*) and methanol dehydrogenase (*mxaF*) which code for key enzymes in the catabolism of all methanotrophs. The yield of PCR products amplified from DNA of soil oxidizing CH_4 was the same as from control soil when using the universal SSU rDNA primer set, but was significantly higher when using primer sets specific for methanotrophs. DGGE patterns, and sequences of major DGGE bands obtained with the universal SSU rDNA primer set showed that the community structure was dominated by non-methanotrophic populations related to *Flavobacterium* and *Bacillus,* and was not influenced by CH_4. The community structure of methanotrophs as monitored with specific primer sets was less complex, and consisted of both type I and type II methanotrophs related to *Methylocystis*, *Methylobacter* and *Methylococcus*. DGGE profiles of PCR products amplified with functional gene primer sets targeting the *mxaF* and *pmoA* genes indicated pronounced community shifts with the beginning of CH_4 oxidation. High CH_4 concentrations stimulated both type I and II methanotrophs in rice field soil at non-saturated water content as detected by both ribosomal and functional gene markers.

Introduction

Methane oxidation by methanotrophic bacteria occurs in soil and aquatic environments thus reducing CH_4 emission, and may be a negative feedback on atmospheric CH_4 increases (17,49). The budget of atmospheric CH_4 is of concern since it is one of the important greenhouse gases, and affects Earth's climate (15,48). Methanotrophs are important regulators of CH_4 emission from rice fields. Only part of the CH_4 produced in the rice field soil is released into the atmosphere, the remainder is oxidized by methanotrophic bacteria living in oxic niches in flooded fields, i.e. the surface soil layer and the rhizosphere (7,18,24, 32,54). Slurries of anoxic rice field soil change from production to consumption of CH_4 when aerated (28). In non-saturated rice field soil CH_4 oxidation is induced when the soil is moistened and exposed to CH_4 concentrations higher than 1000 ppmv (3,4). Most probable number counts of methanotrophic bacteria also increase under these conditions. Type II methanotrophs (belonging to *α-Proteobacteria*) were isolated from rice roots (26) . However, the methanotrophic community structure, and its possible change during the induction of CH_4 oxidation in rice field soil is unknown.

In general, little is known about the methanotrophic community structure in soil, but the presence of type II methanotrophs seems to be more frequently reported than that of type I methanotrophs (27). Immunofluorescent analysis of tundra soils indicated the presence of both type I and type II methanotrophs (61). Phospholipid analysis of soil microorganisms demonstrated the dominance of type II methanotrophs in boreal peatland soil (57). Hybridization analysis of 16S rRNA extracted from Alaskan soil also indicated a dominance of type II methanotrophs (11). Type II methanotrophs were also found to be the dominant group of methanotrophs in peat bogs (20), whereas type I methanotrophs seem to prevail in aquatic environments, such as lake water (27,51) and lake sediments (6).

With the introduction of denaturing gradient gel electrophoresis (DGGE) to microbial ecology, a valuable molecular fingerprinting technique became available for studying microbial community structure (30,43,44,45). DGGE facilitates separation of mixtures of PCR-amplified gene fragments based on sequence differences (46), and allows to analyze large sample numbers simultaneously. Thus, this technique is ideally suited for monitoring dynamics of microbial communities as influenced by environmental changes.

Recently, DGGE analysis applied to oxic agricultural soils incubated with high CH_4 mixing ratios, revealed the presence of type I methanotrophs in soil extracts, but type II methanotrophs in enrichment cultures from the same soil (34,47). These studies employed 16S rDNA primer sets, detecting a wide range of species belonging to the domain *Bacteria*. However, another study was unable to resolve methanotrophs by DGGE using universal 16S rDNA primers (60).

Methanotrophs are a phylogenetically heterogeneous group belonging to the α- or γ-class of *Proteobacteria* (27). Seven genera distinguished in type I (γ-*Proteobacteria*) and type II (α-*Proteobacteria*) methanotrophs have been proposed (8,9). While the genera *Methylococcus* (also classified as type X), *Methylomonas, Methylobacter* and *Methylosphaera* belong to the type I methanotrophs, *Methylocystis*, *Methylosinus* and *Methylomicrobium* compose the type II methanotrophs (27). 16S rDNA probes have been developed that target methanotrophic bacteria belonging to either type I and type II (11,39,59). Hybridization of the total extracted environmental DNA of a blanket peat bog by using genus-specific probes suggested that *Methylosinus* and *Methylococcus* were the dominant methanotrophs in this environment, while *Methylomonas* and *Methylobacter* could not be detected (20).

Methanotrophs share a similar physiology. The key enzymes particulate methane monooxygenase (pMMO), soluble methane monooxygenase (sMMO) and methanol dehydrogenase (MDH) are highly conserved, so that enzyme-based gene markers may offer the possibility to detect all known methanotrophs (27). Gene probes targeting functional genes have been developed for the *pmoA*-gene (40) coding for the α-subunit of the pMMO present in all known methanotrophs and the *mxaF*-gene (37,39) coding for the α-subunit of the MDH present in all methylotrophs (27,38). The pMMO is closely related to the ammonium monooxygenase (AMO) of the ammonium oxidizers, and the degenerate pmoA primer set (A189f/A682r) also detects the homologous sequence of the *amoA* gene for the α-subunit of the AMO (31). Gene probes have also been developed targeting the *mmoB* gene for the sMMO (38) present in most type II methanotrophs and in *Methylococcus* (type X), and deficient in most type I methanotrophs (27). Community analysis of environmental DNA from a blanket peat bog by cloning and sequencing of these gene fragments revealed a distinct phylogenetic cluster of type II methanotrophs representing probably new, yet uncultured acidophilic methanotrophs (37,39,40,38).

We studied changes in microbial community structure during the induction of CH_4 oxidation in rice field soil by DGGE analysis of SSU rRNA-based and functional gene markers. An universal SSU rRNA-based primer set, and two primer sets targeting methylotrophic γ- and α-*Proteobacteria* were used. In addition, we employed primer sets targeting functional genes such as the *pmoA*, *mmoB*, and *mxaF* (39,40,38). This approach facilitated the detection of changes in microbial community structure during the induction of CH_4 oxidation in a non-saturated rice field soil.

Materials and Methods

Soil. The rice field soil used has been described previously in detail (28). The soil had a maximum water-holding capacity (WHC) corresponding to a gravimetric water content of 44% (wt/wt). In the following experiments, rice field soil was moistened with demineralized water to a gravimetric water content of 19% (wt/wt) corresponding to 43% WHC. The moist soil was sieved to 2 mm size to ensure homogeneity and minimize anoxic microsites.

CH_4 oxidation. 4.1 ± 0.1 g (fresh weight) soil was filled into 120-ml serum bottles, and closed with butyl rubber stoppers. Fourteen bottles were flushed with moistened synthetic air (20.5% O_2 in N_2), and subsequently supplemented with 50,000 ppmv CH_4. A second set of 8 bottles was not supplemented with CH_4. All bottles were incubated at 25°C in the dark. Three bottles were used for gas analysis only, while the remaining bottles were used for DNA extraction. Four bottles were replenished three times with 5 ppmv CH_4 after the soil had consumed the supplemented CH_4 below a mixing ratio of about 1.8 ppmv. Bottles not supplemented with CH_4 were used as a control for DNA extraction to provide community data for rice soil not supplemented with CH_4. For DNA extraction rice soil was sampled (one bottle each time) at indicated time points (Fig. 1), and stored at -20°C.

Gas analysis. Methane and O_2 were periodically measured by gas chromatography as described previously (28). Oxygen was repeatedly added to the bottles to keep the O_2 concentration constant at about 17% to 20% (vol/vol).

Bacterial strains. For molecular analyses the methanotrophic reference strains from the collection of our institute were used as previously described by Gilbert et al (1998) (25). The bacteria were cultured in nitrate mineral salts medium at pH 6.8 and 15% CH_4 in the headspace (24). The cultures were harvested after 2-3 days by centrifugation, and the cell pellets were stored at –20°C until DNA extraction.

DNA extraction. DNA extraction from rice field soil, and from pure cultures of methanotrophs was modified after Moré *et al*. (1994) (42). Cell pellets, or 0.6 g soil (fresh weight) were transferred into 2-ml screw cap tubes. Approximately 1 g of sterilized (170°C for 4 h) zirconia/silica beads (0.1 mm diameter; Biospec products Inc., Bartlesville, Ok, USA), 800 µl Na-phosphate buffer (120 mM, pH 8), and 260 µl SDS-solution (10% SDS, 0.5 M Tris/HCl, pH 8.0, 0.1 M NaCl) were added to the soil, and resuspended homogeneously by vortexing. Cells were lysed for 45 s by shaking in a cell disruptor (FP120

FastPrep, Savant instruments Inc., Farmingdale, NY, USA) at a setting of 6.5 m s^{-1}. After centrifugation (3 min at 12,000 × g) the supernatant was collected, and the soil-beads mixture was extracted a second time by resuspension in 700 µl phosphate buffer. Proteins and debris were precipitated from the supernatant by adding 0.4 volumes of 7.5 M ammonium acetate, followed by incubation on ice for 5 min. After centrifugation at 12,000 × g for 3 min nucleic acids were precipitated by addition of 0.7 volumes of isopropanol, followed by centrifugation at 12,000 × g and 4°C for 45 min. Subsequently, the DNA pellet was washed with 70% ethanol at 4°C, and dried under vacuum. Finally, DNA was resuspended in 200 µl Tris-EDTA buffer (10 mM Tris-base, 1 mM EDTA, pH 8). Soil DNA was further purified using the Prep-A-Gene kit (Bio-Rad, Munich, Germany) as specified by the manufacturer.

PCR amplification. For PCR amplification we used three SSU rRNA-based primer sets, i.e. an "Universal" primer set targeting all life which was modified from Weisburg et al. (1991) by using 533f (GTGCCAGCAGCCGCGGTAA) and 907r (AATTCCTTTGAGTTT) (*Escherichia coli* positions 515-533 and 907-922 (10)) as forward and reverse primers (62), respectively, and the primer sets "MB10γ" and "MB9α". The latter two primer sets were formulated by utilizing hybridization-probes 10γ and 9α targeting methylotrophic γ- and α-*Proteobacteria* (59), respectively, as forward primers in combination with primer 533 as back primer. In addition, three functional primer sets, i.e. "pmoA", "mmoB", and "mxaF", originally termed A189f/A682r, 77f/369r, and f1003 /r1561, respectively, were utilized (37,40,38).

The primers and the PCR conditions for each primer pair are summarized in Table 1. PCR buffer (20 mM Tris-HCl, pH 8.3, 50 mM KCl), 1 U *AmpliTaq* DNA polymerase (Perkin Elmer Applied Biosystems, Weiterstadt, Germany), 0.5 µM of each primer, 100 µM of each deoxynucleoside triphosphate (Amersham Life Science, Braunschweig, Germany), and 1 µl of template DNA were added to a total reaction volume of 50 µl at 4ºC. Alternatively, where indicated (Table 1), a MasterAmp 2× PCR premix containing 100 mM Tris-HCl (pH 8.3), $MgCl_2$, 400 µM of each deoxynucleoside triphosphate, and the PCR enhancer betaine (Epicentre Technologies, Madison, WI, USA) was added to the reaction solutions. Amplifications were started by placing cooled (+4ºC) PCR tubes immediately into the preheated (94ºC) thermal block of a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). The thermal cycling profile consisted of an initial denaturation of 3 min at 94ºC, followed by 28 to 32 cycles (depending on the primer set) of 30 s at 94ºC, 30 s at an annealing temperature indicated in Table 1, and 45 s at 72ºC (elongation), and 5 min at 72ºC for the last cycle. For PCR amplification with the primer sets pmoA and Universal, PCR conditions were optimized using a touchdown program.

Aliquots (5 µl) of PCR products were analyzed by electrophoresis on 3% agarose gels, stained with ethidium bromide, and quantified densitometrically. Gels were destained in water for 30 min. For calibration the Smart-Ladder DNA mass and size ruler (Eurogentec, Seraing, Belgium) was used (calibration coefficient of all analyses r > 0.9). Gels were photographed with an imaging system (MWG Biotech, Germany), and DNA bands were analyzed with the RFLP-scan software (CSP Inc., Billerica, Belgium).

Table 1. Phylogenetic primers, functional primers and PCR-DGGE used; GC clamps are indicated by stars *cgcccgccgcgccccgcgcccggcccgccgcccccgcccc
**ccccccccccccgccccccgcccccccgccccgccgccc

Primer	Target gene	Temperature for specificity in PCR (°C)	Reaction mix for PCR	DGGE	Reference
Universal (533f/907r*)	SSU rRNA	touchdown 60-50°C	1.5 mM $MgCl_2$	35-80%; 5 h; 150V	[62]
MB10γ (197f/533r*)	16S rRNA	60°C	premix F	20-70%; 5 h; 150V	[59]
MB9α (142f/533r*)	16S rRNA	60°C	premix F	20-70%; 5 h; 150V	[59]
pmoA (A189**/A682)	*pmo*A	touchdown 62-52°C	premix F	35-80%; 6 h; 200V	[40]
mxaF (f1003/r1561**)	*mxa*F	55°C	premix E	35-70%; 5 h; 150V	[39]
mmoB (f77/369r)	*mmo*B	59°C	1.5 mM $MgCl_2$	n.d	[38]

Cloning. 16S rDNA PCR products of soil DNA amplified with the MB9α primer set were cloned using the pGEM-TEasy cloning kit (Promega, Madsion, WI). Randomly selected clones were sequenced as described previously (52). Cloned inserts were amplified by primers targeting vector sequences. For DGGE analysis of MB9α clones, PCR products were amplified with the MB9α primer set.

DGGE. DGGE was carried out as described previously in detail with minor modifications (44). PCR products were separated using a DCode System (Bio-Rad, Munich, Germany) on 1-mm thick polyacrylamide gels (6.5% w/v acrylamide:bis acrylamide (37.5:1); Bio-Rad) prepared with and electrophoresed in 0.5 × TAE, pH 7.4 (0.04 M Tris-base, 0.02 M sodium-acetate, 1 mM EDTA) at 60°C, and constant voltage. A denaturing gradient of 80% (vol/vol) denaturant corresponded to 6.5% acrylamide, 5.6 M urea and 32% deionized formamide. Gels were poured on GelBond PAG film (FMC Bioproducts, Rockland, ME, USA) to avoid gel distortion. DGGE conditions for the various PCR products were optimi-

zed by perpendicular DGGE (43). Running conditions are summarized in Table 1. Gels were stained with 1:50,000 (vol/vol) SYBR-Green I (Biozym, Hessisch-Oldendorf, Germany) for 30 min, and scanned with a Storm 860 phosphor imager (Molecular Dynamics, Sunnyvale, CA, USA). Some gels were silver-stained (12), dried, and documented using an overhead scanner (Scanjet 4c/T, Hewlett-Packard, Palo Alto, CA, USA).

Extraction of PCR products from DGGE gels. Due to its spectral characteristics SYBR Green I bound to dsDNA is maximally excited at 497 nm, and fluorescence emission is centered around 520 nm wavelength (Molecular Probes, Eugene, OR; http:\\www.probes.com handbook/figs/fig08-10.html). Thus, dsDNA in gels can be detected with non-UV light sources which is a prerequisite to avoid UV light-induced DNA damage. We visualized DGGE bands in SYBR Green I-stained gels with blue light ($\lambda > 400$ nm) using a Dark Reader transilluminator (Clare Chemical Research, Ross on Wye, UK). Individual DGGE bands were sampled by excising a small core with a sterile 200 µl pipette tip, reamplified, and reanalyzed by DGGE to verify band purity.

Sequencing of DGGE bands. Reamplified PCR products of excised DGGE bands were purified using the EasyPure DNA purification kit (Biozym, Hessisch-Oldendorf, Germany). Concentration and purity of PCR products were determined by absorption at 260 nm and 280 nm of a 1:20 dilution in H_2O with a GeneQuant spectrophotometer (Pharmacia Biotech, Upsala, Sweden). Sequencing reactions were performed using the ABI Dye-terminator cycle sequencing kit (Perkin Elmer Applied Biosystems,) with 30-180 ng template DNA as specified by the manufacturer. Cycle sequencing products were purified from excess dye terminators and primers using Microspin G-50 columns (Pharmacia Biotech, Freiburg, Germany), and analyzed with an ABI 373 DNA sequencer (Perkin Elmer Applied Biosystems).

Sequences were analyzed using the Lasergene software package (DNASTAR, Madison, WI, USA). Nucleotide and inferred amino acid sequences of the gene fragments of *pmoA* and *mxaF* were manually aligned with sequences retrieved from the GenBank database. 16S rDNA sequences were aligned and phylogenetically placed with the ARB software package (56). On the nucleic acid level, evolutionary distances between pairs of sequences were calculated by using the Jukes-Cantor, and Felsenstein equations (22,35) implemented in the ARB package. Phylogenetic trees were constructed by using the neighbor joining algorithm supplied by the ARB software package (56).

Nucleotide accession numbers. Sequences of partial *pmoA* and *mxaF* gene fragments, and of 16S rRNA gene fragments of excised DGGE bands have been deposited in GenBank under accession no. AF126295 to AF126297, and Af126908 to AF126945.

Results

Induction of CH_4 oxidation in rice field soil. When moist rice field soil was incubated in the presence 50,000 ppmv CH_4, oxidation started after a lag phase of about 24 h. Methane decreased linearly for approximately 48 h, then a second phase of faster CH_4 consumption followed (Fig. 1). The supplied CH_4 was consumed to a residual concentration of 1 ppmv, i.e. below the atmospheric mixing ratio of about 1.8 ppmv (Fig. 1).

After the initial consumption below 1.8 ppmv CH_4, CH_4 was added again to a concentration of about 6 ppmv, which was subsequently oxidized, but to a residual concentration higher than ambient (Fig. 1). Subsequent additions of CH_4 resulted in a steadily decreasing CH_4 oxidation activity which eventually ceased. The CH_4-oxidizing populations were apparently not able to maintain their activity when supplied with low (5-6 ppmv) concentrations of CH_4 for a prolonged period (Fig. 1).

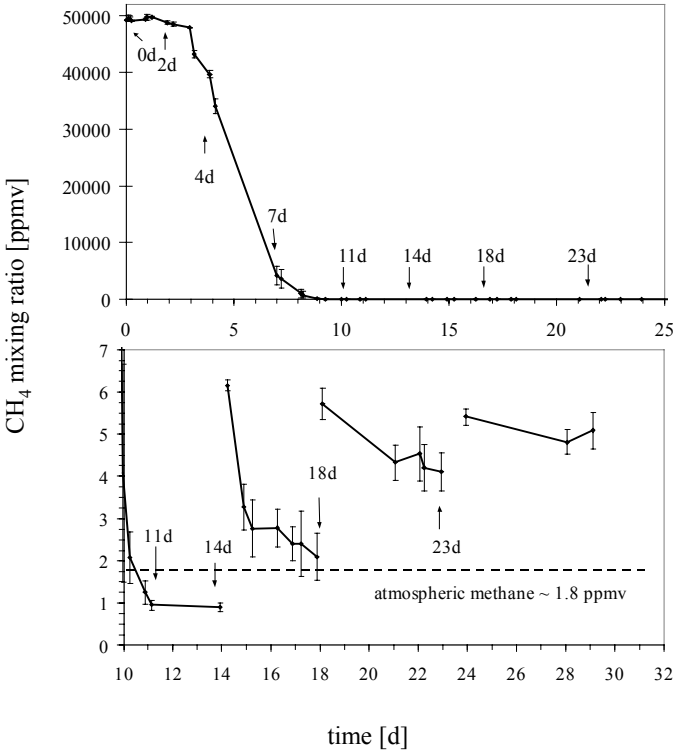

Figure 1. Oxidation of CH_4 at mixing ratios of 50,000 ppmv in bottles containing rice field soil at 43% WHC. CH_4 (5-6 ppmv) was replenished at days 14, 18 and 24 after the initial CH_4 mixing ratio had been consumed below atmospheric CH_4 level. Soil samples for molecular analyses were taken at time points indicated; mean ±SD, n = 3.

PCR amplification. Soil samples were taken at time points indicated in Fig. 1 from CH_4-supplemented soil, and DNA was extracted for PCR amplification. Soil samples were periodically also taken from controls to which no CH_4 was added. PCR products of expected sizes were obtained by amplification with functional primer sets pmoA and mxaF, and

16S rDNA primer sets MB9α, MB10γ and Universal, using template DNA isolated from 9 different methanotrophic reference strains, and from soil samples. PCR products of correct size were also obtained with primer set mmoB targeting the *mmoB* gene coding for the β-subunit of sMMO for the appropriate reference strains. However, only a very weak PCR product was obtained from soil samples oxidizing CH_4. PCR amplification with primer set mmoB containing a GC-clamp failed, and thus, DGGE analysis of *mmoB* PCR products was not possible.

PCR amplification with the functional primer sets pmoA, mxaF, and the 16S rDNA primer sets MB9α and MB10γ yielded significantly lower PCR product concentrations from template DNA extracted from control soil, or lag-phase soil (0 d and 2 d after adding CH_4) than from soil during the phase of vigorous CH_4 consumption (4 d – 23 d) (Fig. 2). In contrast, PCR amplification with the Universal SSU rDNA primer set resulted in similar PCR product yields irrespective of the soil sample (Fig. 2). The uniform PCR amplification yield of all soil samples with the Universal primer set suggested that PCR-inhibiting substances in DNA templates, such as humic acids (58), did not bias amplification.

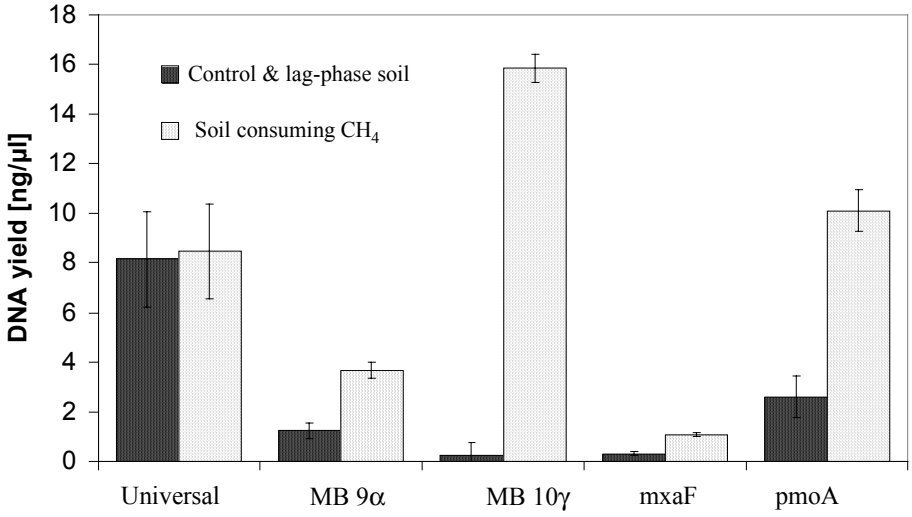

Figure 2. PCR yields of amplifications with different primer sets, and template DNA isolated from soil samples without CH_4 oxidation (i.e. dry soil, control soil, lag phase soil of days 1 and 2), and with CH_4 oxidation (days 4 to 23); mean ± SD, n = 6-7.

SSU rDNA DGGE and sequence analysis of of *Bacteria*. DGGE analysis of PCR products amplified with the Universal primer set revealed a complex banding pattern, which was similar for control soil without CH_4 and soil supplemented with CH_4 (Fig. 3). DGGE profiles of dry rice field soil and control soil until 8 d of incubation exhibited fewer DGGE bands than at later sampling events. The number and intensity of bands increased after the soil was moistened. CH_4-supplemented soil at day 0 also showed fewer DGGE bands

than at later sampling events. Sequences of major DGGE bands (bands I to V, Fig. 3) grouped either within the Gram-positive branch close to *Bacillus* species, or within the *Cytophaga-Flavobacterium-Bacteroides* (CFB) kingdom (Fig. 3). However, sequences of methanotrophic or methylotrophic bacteria could not be detected. Slight temporal changes in banding patterns (Fig. 3) probably reflected CH_4-independent activation of bacteria by the increased soil water content. The observation that even Gram-positive, spore-forming *Bacillus* species known as notoriously recalcitrant to lyse were detected by DGGE indicated that lysis and DNA extraction protocols were effective (42,41).

16S rDNA DGGE and sequence analysis of type I methylotrophs. DGGE profiles of PCR products amplified with the MB10γ primer set showed three major bands, demonstrating that type I methanotrophs were less diverse than the total bacterial community (Fig. 4). PCR product yields obtained from control and lag phase soil (sampling 0 d and 2 d after CH_4 addition) were consistently lower than those obtained from soil samples consuming CH_4 (Fig. 4) confirming PCR product yields shown in Fig. 2. Although twice the volume of PCR product was loaded onto DGGE gels, band intensities with control soil were still lower than those with CH_4-oxidizing soil (Fig. 4, sampling after 4 d to 23 d). Three bands were retrieved successfully from DGGE gels. DNA sequences of these bands grouped closely to *Methylobacter* species within the radiation of type I methanotrophs (γ-*Proteobacteria*) (Fig. 4).

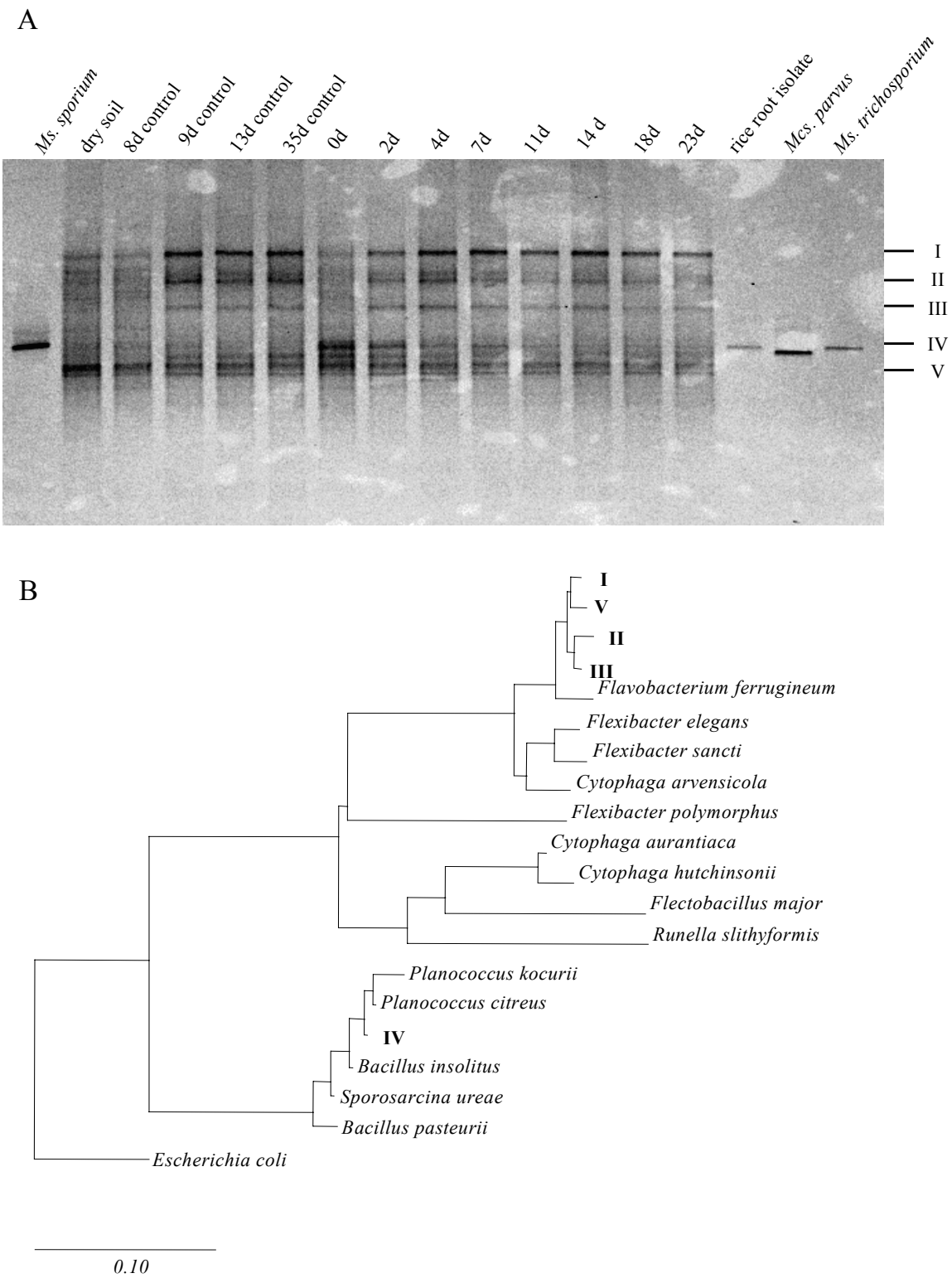

Figure 3. (A) DGGE banding pattern obtained with the Universal 16S rDNA primer set targeting all life. 20 µl ≅ 160 ± 41 ng of PCR product for all soil samples. Marked bands were sequenced. *Mcs, Methylocystis*; *Ms, Methylosinus*. (B) Phylogenetic tree constructed with partial 16S rDNA sequences showing the relationship of the labeled DGGE bands to closely related *Bacteria*. The scale bar represents the estimated number of base changes per nucleotide sequence position.

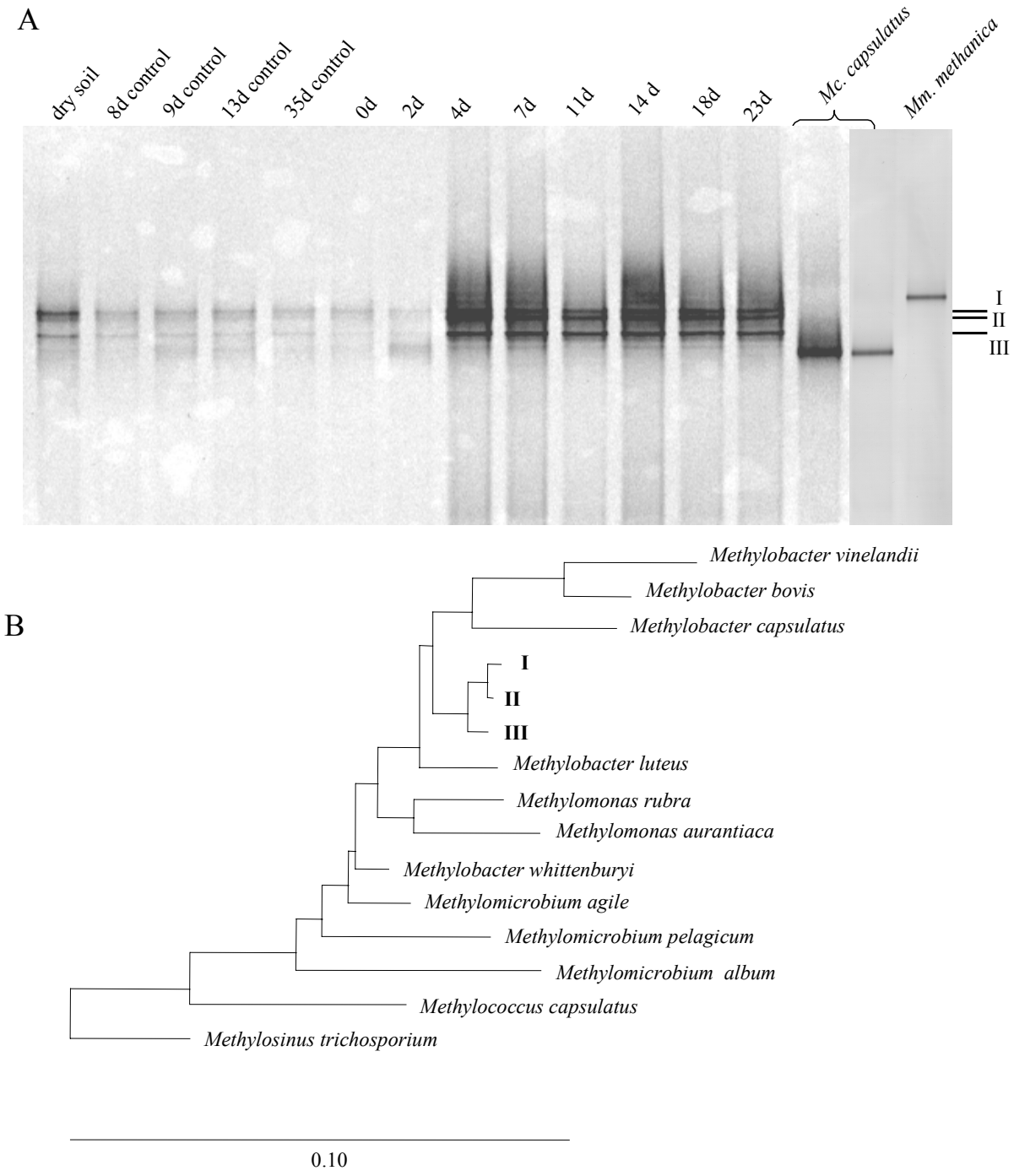

Figure 4. (A) DGGE banding pattern obtained with the 16S rDNA primer set MB10γ targeting type I methanotrophs. For soil without CH_4 oxidation (i.e. dry soil to 2d) 40 µl (≅ 63-100 ng) of PCR product, and for soil with CH_4 oxidation (i.e. 4d to 23d) 25 µl (≅ 397 ± 80 ng) of PCR product were loaded on each lane. *Mc, Methylococcus*; *Mm, Methylomonas*. (B) Phylogenetic tree based on 16S rDNA sequences showing the relationship of the labeled DGGE bands to the most closely related γ-*Proteobacteria*. The scale bar represents the estimated number of base changes per nucleotide sequence position.

16S rDNA DGGE and sequence analysis of type II methanotrophs. DGGE banding patterns of MB9α PCR products showed about 12 major bands after incubation with CH_4. Control soil samples showed about 7 major bands, of which all were also present in the soil oxidizing CH_4 (Fig. 5, sampling after 18 d and 23 d). Band intensities of 6 bands increased after the soil had started to consume CH_4 (Fig. 5) analogously as for the MB10γ PCR products. Excised and re-amplified MB9α DGGE bands could not be retrieved purely from DGGE gels as indicated by the appearance of several DGGE bands when rerun on a DGGE gel. Therefore, the MB9α PCR products of control soil (after 13 d), and soil consuming CH_4 (after 14 d) were cloned to obtain sequence data of type II methanotrophs (n=30). Electrophoretic mobilities of PCR products of MB9α clones were similar to those of original soil DGGE bands. However, several DGGE bands of clones did not correspond to the original DGGE banding pattern indicating that community analyses by cloning and by DGGE were subjected to a different bias (results not shown).

All clone sequences grouped within the α-*Proteobacteria*, but only 1/3 of the sequences grouped within the type II methanotrophs, i.e. *Methylosinus* and *Methylocystis* species (Fig. 5). The other clones grouped with *Caulobacter* and *Sphingomonas* (Fig. 5; not all clones are shown). Two clone sequences (clones c4 and c5) grouped closely with *Beijerinkia indica*. An acidophilic methanotroph (strain S6) recently isolated from a peat bog in Russia, and probably representing a novel methanotrophic group (19), was also closely related to *Beijerinkia,* and showed 97% similarity to clones c4 and c5 of rice field soil.

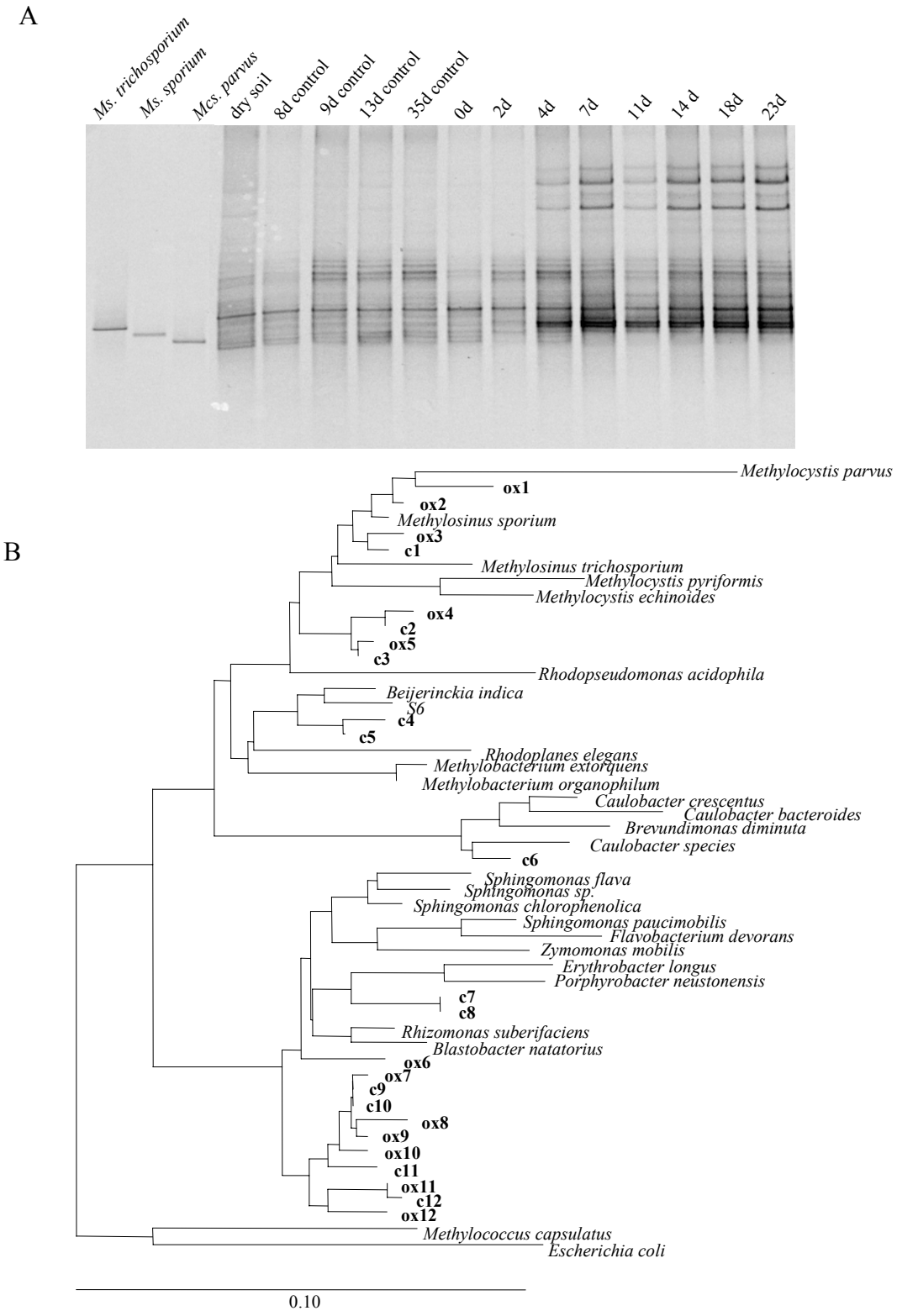

Figure 5. (A) DGGE banding pattern obtained with the 16S rDNA primer set MB9α targeting type II methanotrophs. For soil without CH_4 oxidation (i.e. dry soil to 2d) 40 µl (≅ 360 ± 118 ng) of PCR product were loaded on each lane, and for soil with CH_4 oxidation (i.e. 4d to 23d) 40 µl (≅ 572 ± 162 ng) of PCR product. *Mcs, Methylocystis*; *Ms, Methylosinus*. (B) Phylogenetic tree based on 16S rDNA sequences showing the relationship of MB9α clones to the most closely related α-*Proteobacteria*. The scale bar represents the estimated number of base changes per nucleotide sequence position. c, clones from control soil (after 13d); ox, clones from soil with CH_4 oxidation (after 14d).

DGGE and sequence analysis of the pMMO gene. Several methanotrophs contain at least two pMMO gene copies (55). This could explain the appearance of multiple strong DGGE bands for the pmoA PCR product of *Methylococcus capsulatus*, whereas *Methylosinus trichosporium* showed a very faint second band, visible on silver stained DGGE gels only (Fig. 6). DGGE profiles of pmoA PCR products were markedly different in control soil and soil supplemented with CH_4 (Fig. 6). Control soil showed only one major band, whereas CH_4-oxidizing soil exhibited at least 6 additional bands which appeared after 4-7 d of incubation, and increased in intensity. At this time, the soil was oxidizing CH_4 with maximal rate. Simultaneously, the intensity of bands in control soil decreased.

Deduced amino acid *pmoA* sequence data of major DGGE bands confirmed a shift in population structure. The sequence of control soil DGGE band I (Fig. 6) (sampling of dry soil at day 0) indicated an affiliation with ammonium oxidizers, closely related to *Nitrosospira* species (*β-Proteobacteria*) (Fig. 6). On the other hand, sequences of five DGGE bands obtained with CH_4-consuming soil (sampling after 4–23 d) were affiliated with type I and type II methanotrophs. Three sequences grouped most closely with *Methylocystis*, while two type I sequences grouped with *Methylococcus capsulatus*.

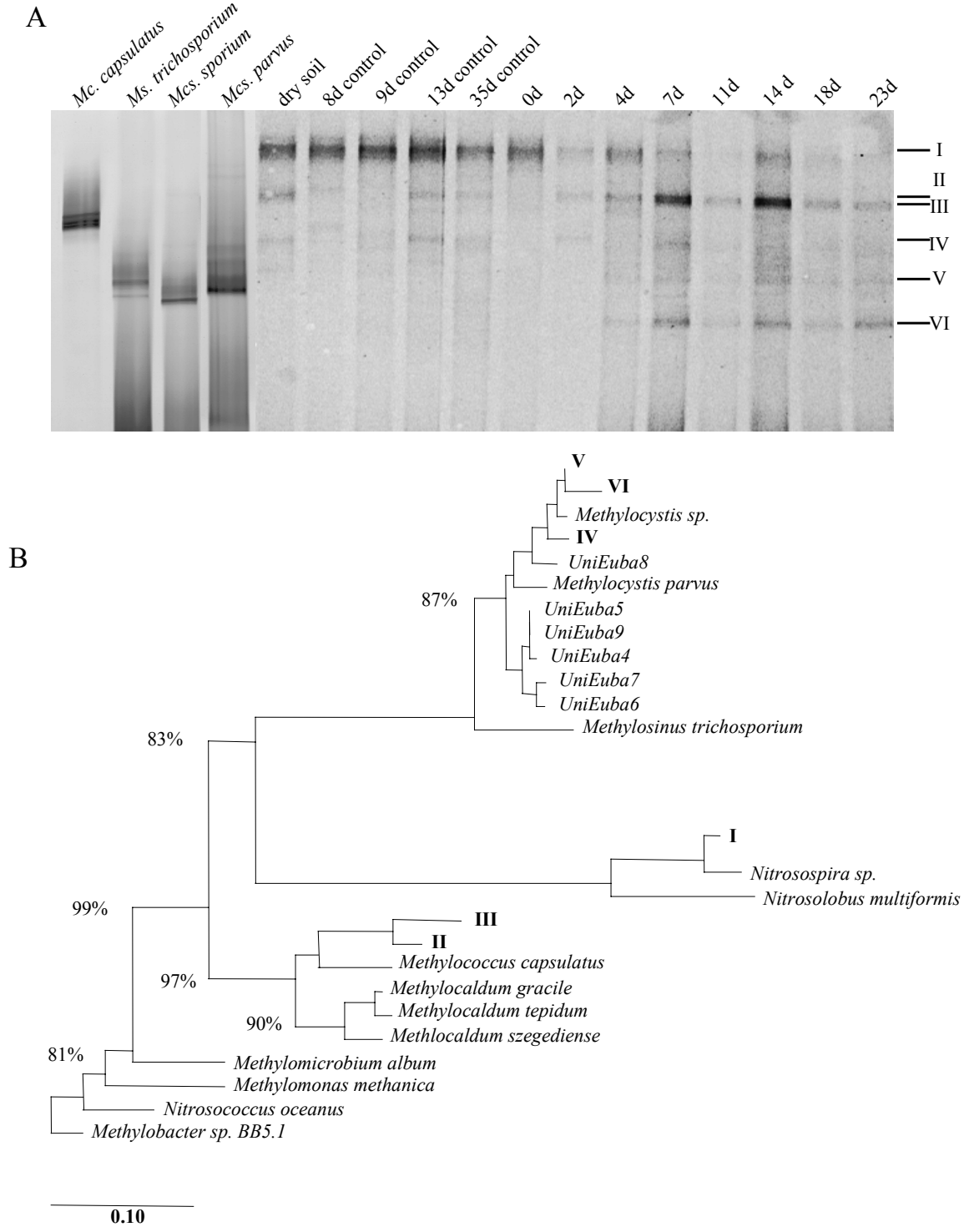

Figure 6. (A) DGGE banding pattern obtained with the primer set pmoA targeting the gene of the pMMO. 35 µl (≅ 181 ±59 ng) of PCR product from soil without CH_4 oxidation (i.e. dry soil to 2d), and 35 µl (≅ 707± 203 ng) of PCR product from soil with CH_4 oxidation (i.e. 4d to 23d) were loaded on each lane. *Mc, Methylococcus; Ms, Methylosinus*; *Mcs, Methylocystis*. (B) Phylogenetic tree based on derived amino acid sequences of *pmoA* fragments, showing the relationship of the labeled DGGE bands to *pmoA* sequences of other methanotrophs. The scale bar represents the estimated number of changes per amino acid sequence position. Bootstrap values are given for 1000 replicate trees.

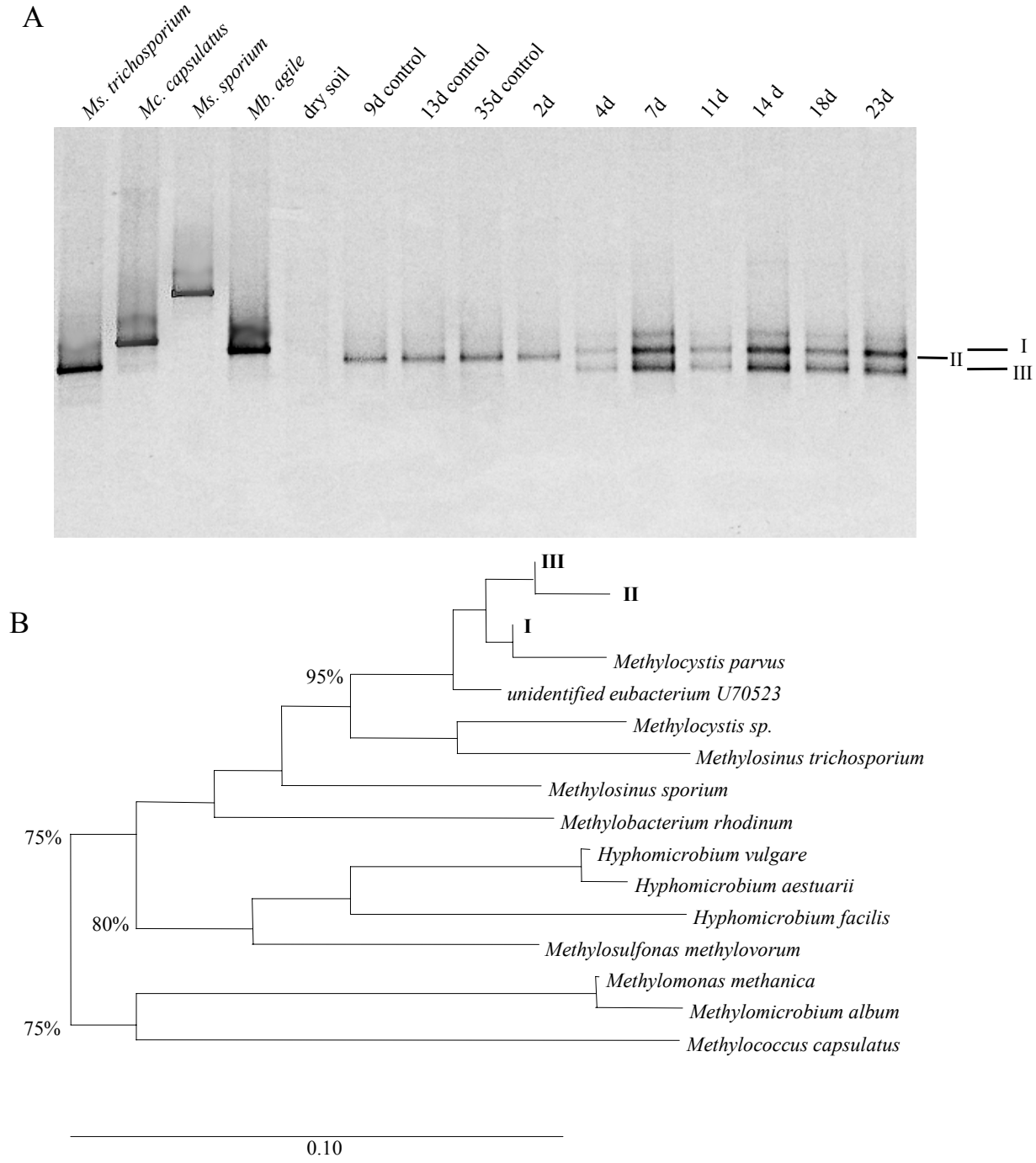

Figure 7. (A) DGGE banding pattern obtained with the primer set mxaF targeting the gene of the MDH. 35 µl (≅ 22 ± 5 ng) of PCR product from soil without CH_4 oxidation (i.e. dry soil to 2d), and 35 µl (≅ 75 ± 56 ng) of PCR product from soil with CH_4 oxidation (i.e. 4d to 23d) were loaded on each lane. *Mc, Methylococcus*; *Ms, Methylosinus*; *Mb, Methylobacter*. (B) Phylogenetic tree based on derived amino acid sequences of *mxaF* fragments, showing the relationship of the labeled DGGE bands to *mxaF* sequences of other methylotrophs. The scale bar represents the estimated number of changes per amino acid sequence position. Bootstrap values are given for 1000 replicate trees.

DGGE and sequence analysis of the MDH gene. *MxaF* PCR products amplified from DNA extracted from control soil, and soil supplemented with CH_4 were very weak and sometimes even undetectable at the beginning of the incubation. Control soil exhibited only one DGGE band, whereas CH_4-supplemented soil displayed three bands after the onset of CH_4 oxidation, i.e. 4 d after the CH_4 supplementation (Fig. 7). Interestingly, the band in the control soil apparently was absent in soil consuming CH_4 (Fig. 7). *MxaF* gene sequences of purified DGGE bands of control soil and of soil consuming CH_4 both grouped within the cluster of type II methanotrophs (Fig. 7)

Discussion

Rice field soil moistened to 43% WHC, and initially supplemented with 5% CH_4, oxidized CH_4 after a lag phase. The soil then consumed CH_4 below atmospheric levels, but was unable to maintain this capacity for a prolonged period of time if only supplemented with low CH_4 mixing ratios (< 6 ppmv). We assume that the initial CH_4 oxidation activity (V_{max}) was high enough to allow the consumption of CH_4 at atmospheric trace gas concentrations, but later on ceased, probably because the oxidation of low CH_4 concentrations did not allow to generate the maintenance energy necessary for enzyme synthesis (16). Similar observations of decreasing CH_4 oxidation rates after induction of CH_4 oxidation at high CH_4 concentrations have been made in other soils (4,50,53).

The molecular analysis of the soil microbial community during incubation in the presence of CH_4 showed the following: (i) PCR amplification with the specific 16S rDNA primer sets MB10γ, and MB9α, as well as with the functional primer sets pmoA and mxaF resulted in significantly higher PCR product concentrations from soil consuming CH_4 than from control soil; (ii) DGGE bands of MB10γ, MB9α, pmoA and mxaF PCR products increased in intensity when soil consumed CH_4; (iii) DGGE analysis of pmoA, mxaF, and MB9α PCR products revealed differences in banding patterns between control soil and CH_4-consuming soil.

We hypothesize that the higher PCR product concentrations observed for amplification with specific primer sets MB10γ, MB9α, and primers targeting *pmoA* and *mxaF* from soil consuming CH_4 indicate more target sites in soil oxidizing CH_4 than in control soil due to stimulation by CH_4. It is unlikely that the observed differences in PCR product concentrations were due to different concentrations of PCR-inhibiting compounds such as humic acids in the template DNAs extracted from soil. Since all soil samples were treated equally for DNA extraction, potential PCR inhibitors should *a priori* have the same concentration in all soil samples. This presumption was supported by PCR amplifications with the Universal primer set. These amplifications showed the same yield of PCR product regardless of the soil sample.

The determination of microbial abundance solely based on the PCR product concentrations is impossible (13,21). However, Ferris & Ward (1997) concluded that a change in the intensity of a particular DGGE band in a temporal or spatial environmental gradient may be used to infer changes in size of the respective populations (23). However, intensities of different bands are not comparable to each other. Therefore, we conclude that the observed increase in intensity of certain DGGE bands with longer exposition of soil to CH_4 indicates an increase of a respective methanotrophic population. Since the intensity of DGGE bands amplified with primers targeting genes specific for methanotrophs were higher in the presence than in the absence of CH_4, our data suggest that the abundance of CH_4-oxidizing populations had increased in rice field soil after receiving high mixing ratios of CH_4. Such an increase of methanotrophic populations has also been demonstrated by most probable number counts using incubations with initial CH_4 mixing ratios of > 7000 ppmv CH_4 (4).

The DGGE banding pattern of 16S rDNA templates amplified with the Universal primer set was as complex as expected for a habitat such as soil. Changes in DGGE banding pattern occurred only at the beginning of the experiment when soil water content changed from dry to moist. Addition of CH_4, however, had no influence on the DGGE banding pattern. We assume that the soil microorganisms responded to the changed soil water content and caused population shifts. DNA sequences of major DGGE bands grouped closely to *Flexibacter*, *Flavobacterium* within the CFB kingdom, and to *Bacillus* within the Gram-positive bacteria with low G+C content. Isolation of fermentative abundant rice field soil bacteria , and a molecular survey of microbial diversity by cloning of 16S rDNA genes showed that indeed bacteria of these two kingdoms represent a major part of the bacterial community in rice field soil (14,29).

The DGGE banding patterns of PCR products obtained with the 16S rDNA primer sets MB10γ and MB9α targeting methanotrophic bacteria, as well as functional primer sets targeting *pmoA* and *mxaF* showed fewer bands than those obtained with the Universal SSU rDNA primer set. This observation is consistent with the assumption that the community of specialized CH_4 oxidizers was smaller than the community of *Bacteria* in general. DNA sequences of MB10γ and pmoA DGGE bands grouped closely to *Methylobacter* sp., and *Methylococcus capsulatus* within the type I methanotrophs. However mxaF DGGE bands, some pmoA DGGE bands, and clone sequences of MB-9α grouped within the type II methanotrophs. The presence of type II methanotrophs (or of type X, e.g. *Methylococcus capsulatus*) in soil consuming CH_4 was also shown by PCR targeting *mmoB*, but only soil samples oxidizing CH_4 at high rates exhibited a *mmoB* PCR product.

Differences between DGGE banding patterns of soil incubated in the presence and absence of CH_4 were observed with pmoA and mxaF PCR products. The major DGGE

band detected and sequenced in control soil grouped closely to *Nitrosospira* species. The primer set pmoA targeting *pmoA* also amplifies the *amoA* gene of ammonium oxidizers coding for the α-subunit of the ammonium monooxygenase (31). PmoA DGGE bands, that had been verified by sequencing as related to methanotrophs, intensified after the onset of CH_4 oxidation, while the pmoA DGGE band closely related to *Nitrosospira* became fainter. Apparently, ammonium-oxidizing populations were outnumbered or out-competed by methanotrophs when CH_4 consumption started. Our observation is in agreement with recent studies by Bodelier & Frenzel (5) who were able to demonstrate that ammonium oxidizers did not contribute to CH_4 oxidation in rice microcosms

16S rDNA sequence analysis of clones revealed that the MB9α primer set was not specific for type II methanotrophs, and detected other *α-Proteobacteria* as well. Therefore, changes of DGGE banding pattern observed with MB9α PCR products after the onset of CH_4 oxidation cannot be linked to methanotrophs exclusively. However, 16S rDNA sequences that clustered with methanotrophic bacteria were related to type II methanotrophs, thus supporting the data based on mxaF and pmoA analysis. The number, and diversity of sequence types obtained by cloning of MB9α PCR products indicated a higher diversity than that observed by DGGE analysis of the same PCR product. DGGE analysis revealed only the most abundant populations, while cloning probably detected also less abundant populations.

Two MB9α clones grouped closely with *Beijerinkia indica* and novel, recently isolated, acidophilic methanotrophs (19). The detection of these closely related populations in neutral rice field soil suggests that this new group of methanotrophs may not be limited to acidic peat bogs. However, isolation of these novel methanotrophs from rice field soil is mandatory to support our molecular finding.

Our knowledge of the community structure of methanotrophs in rice field soil has been limited. Recently, two type II methanotrophs (strains Rp1 and Rp2) were isolated from high dilutions of MPN counts from the rhizoplane of rice roots (26). Generally, type II methanotrophs have been more frequently detected in soil environments than type I methanotrophs (see Introduction). Several factors influencing competition between type I and type II methanotrophs such as CH_4 and O_2 concentrations, nitrogen and copper availability are currently being discussed (27). Amaral & Knowles (1, 2) studied the distribution of soil and sediment methanotrophs in agarose diffusion columns with opposing gradients of CH_4 and O_2. They concluded that type I methanotrophs may be favored at low CH_4 and high O_2 concentrations, whereas type II methanotrophs may be favored at high CH_4 and low O_2 concentrations (2). Supportive of this hypothesis is the prevalence of type II methanotrophs in soils and type I methanotrophs in aquatic sediments (27). With respect to O_2 and CH_4 availability flooded rice field soil provides at least two different niches for methanotrophs: (i)

the soil surface of flooded rice field soil (bulk soil), and (ii) the rhizosphere. The soil surface is comparable to aquatic sediments and characterized by steep opposing O_2 and CH_4 gradients (25). At the interface of these gradients, concentrations of O_2 and CH_4 are both very low, which could favor both types of methanotrophs. In the rhizosphere, O_2 and CH_4 concentrations can both be very low, but this compartment is characterized by spatial and temporal heterogeneity of O_2 and CH_4 concentrations due to the influence of the rice roots (25). *In situ*-probing with 16S rDNA-based probes suggested a numerical dominance of type II methanotrophs in the rhizophere of aquatic macrophytes (36), and two numerically relevant type II methanotrophs were isolated from the rhizoplane of rice (26).

In this study, we used rice field soil originating from all these habitats. Our results show that both type I and type II methanotrophs were present in the original rice field soil. When the soil was incubated under moist conditions with high mixing ratios of CH_4, DGGE bands of both type I and type II methanotrophs developed thus indicating that these bacteria became active. Apparently, other factors than O_2 and CH_4 availability may determine the composition of the methanotrophic community in rice field soil.

The active methanotrophs were apparently not able to consume atmospheric CH_4 mixing ratios, since CH_4 oxidation activity eventually ceased, when CH_4 was only supplied at low concentrations (< 5 ppmv). Nevertheless, the populations of methanotrophs seemed to persist as indicated by the DGGE analyses. Recent experiments showed that some CH_4 production takes place even in drained, non-saturated rice field soil, probably since anoxic niches with active methanogenesis persist (33). Therefore, it is possible that methanotrophs were supplied with additional CH_4 that was produced inside of soil aggregates.

Acknowledgments

We thank Bianca Wagner for excellent technical assistance. This work was supported by the European Commission (BIO-4-CT-960419)

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2. Der Einfluss von O_2 und CH_4 auf die Struktur und Aktivität der methanotrophen Lebensgemeinschaft im Reisfeldboden

In dieser Arbeit wurde untersucht, wie unterschiedliche O_2 und CH_4 Mischungsverhältnisse die Zusammensetzung der methanotrophen Lebensgemeinschaft im Reisfeldboden beeinflussen.

Hohe O_2 Mischungsverhältnisse (20,5 %) hemmten das Wachstum und die Aktivität der methanotrophen Lebensgemeinschaft. Wohingegen niedrige O_2 Mischungsverhältnisse (1 %) weder Aktivität noch Biomassezuwachs der methanotrophen Lebensgemeinschaft einschränkte. Auf die Populationsstruktur der Typ I und Typ II MOB wirkte sich die Verfügbarkeit von O_2 und CH_4 unterschiedlich aus. Die Populationsstruktur der Typ I MOB veränderte sich unter den verschiedenen O_2 / CH_4-Atmosphären sehr stark und mit einer hohen Dynamik, während sich die Populationsstruktur der Typ II MOB nur geringfügig veränderte. Sowohl CH_4 als auch O_2 beeinflussten die Diversität und Zusammensetzung der Populationsstruktur von Typ I MOB. Unter niedrigen CH_4 Mischungsverhältnissen dominierten Typ I MOB gegenüber Typ II MOB.

Submitted to Environmental Microbiology

Effects of O_2 and CH_4 on presence and activity of the indigenous methanotrophic community in rice field soil

Thilo Henckel, Peter Roslev and Ralf Conrad

Abstract

Methanotrophs are important in controlling CH_4 emission from wetland rice fields. Their activity and distribution depend on the availability of CH_4 and O_2, but the effect of different CH_4 and O_2 concentrations on the methanotrophic community is not known. Therefore, we investigated the activity and structure of the methanotrophic community in rice field soil under 4 factorial combinations of high and low CH_4 and O_2 concentrations. Rice field soil at non-saturated water content was incubated under a continuos flow of an artificial atmosphere containing CH_4 at either low (1000 ppmv) or high (**>**10,000 ppmv) plus O_2 at either low (1%) or high (20.5%) mixing ratio. The methanotrophic population structure in the four different incubation set-ups was resolved by PCR-DGGE with 5 different PCR primer sets targeting a universal region and two regions more specific for methylotrophic α- and γ-*Proteobacteria* on the 16S rRNA gene, and two functional genes coding for key enzymes in methanotrophs, i.e. the particulate methane monooxygenase (*pmo*A) and the methanol dehydrogenase (*mxa*F). Changes in the biomass of type I and II methanotrophic bacteria in the rice soil was determined by analysis of the PLFA biomarkers 16:1ω8c and 18:1ω8c, respectively. The relative contribution of type I and II methanotrophs to the measured methane oxidation activity was determined by labelling of soil samples with $^{14}CH_4$ followed by analysis of radioactive phospholipid ester-linked fatty-acids (^{14}C-PLFAs). CH_4 oxidation was repressed by high O_2 (20.5%), and enhanced by low O_2 (1%). Depending on the CH_4 and O_2 mixing ratios different methanotrophic communities developed with a higher diversity at low than at high CH_4 concentration as revealed by PCR-DGGE. However, a prevalence of type I or II populations was not detected. The ^{14}C-PLFA fingerprints revealed that CH_4 oxidation activity was dominated by type I methanotrophs in incubations with low CH_4mixing ratios (1000 ppmv) and during initiation of CH_4 consumption regardless of O_2 or CH_4 mixing ratio. At high methane mixing ratios (10,000 ppmv), type I and II methanotrophs contributed equally to the measured CH_4 metabolism. Collectively, type I methanotrophs responded fast and with pronounced shifts in population structure and dominated activity under all four gas mixtures. Whereas, type II methanotrophs, although apparently more abundant, always present and showing a largely stable population structure, became active later and contributed to CH_4 oxidation activity mainly under high CH_4 mixing ratios.

Introduction

Rice fields are one of the most important sources of the greenhouse gas CH_4. Flooded rice fields (wetland rice) contribute up to 25% to the global CH_4 budget (Neue, 1997; Conrad,1997). Basic knowledge of the underlying processes is required when designing strategies to mitigate and control CH_4 emission from wetland rice fields.

Microbial CH_4-oxidation, by obligate aerobic methanotrophs, is the only terrestrial sink for CH_4. Methanotrophs play an important role in regulating the CH_4 flux from wetlands (Reeburgh et al., 1993; Conrad, 1996). In wetland soils, CH_4 and O_2 are proximal factors influencing methanotrophs (Schimel et al., 1993). The availability of O_2 and CH_4 determines CH_4 oxidation and presumably plays a vital role in the determination of the methanotrophic community. Wetland soils are characterized by steep gradients of O_2 and CH_4. Up to 90% of the produced CH_4 is oxidized by methanotrophs at oxic-anoxic interfaces in the rhizosphere and at the soil-surface layer before escaping to the atmosphere (Conrad and Rothfuss 1991; Frenzel et al., 1992; Gilbert & Frenzel, 1995; King, GM, 1996; van der Gon et al., 1996; Bosse & Frenzel, 1997, 1998;). When rice fields are drained prior to harvest, or intermittently to mitigate CH_4 emission, methanotrophs become also active in the subsoil (Henckel et al., in prep). Soil water content directly influences O_2 availability in soils, and thus is an important factor controlling CH_4 oxidation of rice field soil (Bender & Conrad, 1995). The aerenchyma, a vascular system of air-filled space inside of wetland plants, allows O_2 to diffuse into the rhizosphere. In the soil adjacent to the rice roots the number of methane-oxidizing bacteria increased significantly (Bosse & Frenzel, 1997).

Both type I and type II methanotrophs are present in rice field soil (Henckel et al., 1999). Both types of methanotrophs were activated upon incubation with high CH_4 mixing ratios (Henckel et al., 1999). Amaral and Knowles (1995) reported that type I and type II methanotrophs show an apparent prevalence to the availability of O_2 and CH_4. In agar diffusion columns with counter gradients of O_2 and CH_4, type I methanotrophs dominated in layers with low CH_4 and high O_2 mixing ratios, whereas type II methanotrophs favored high CH_4 and low O_2 mixing ratios (Amaral & Knowles, 1995; Amaral et al., 1995). Oxygen was also identified as the key factor controlling CH_4 oxidation and the distribution of methanotrophs in a stratified lake. Maximal CH_4 oxidation rates were found at 0.1-1.0 mg l^{-1} O_2, while CH_4 oxidation was inhibited by O_2 mixing ratios exceeding 1 mg l^{-1} (Rudd & Hamilton, 1975). Oxygen sensitivity has also been observed in pure cultures of methanotrophs (de-Bont & Mulder, 1974; Whittenbury et al., 1975; Dalton & Whittenbury, 1976). Because of the observed O_2 sensitivity methanotrophs were termed ”functional facultative microaerophils” (Rudd &Taylor, 1980; Hanson & Hanson, 1996).

A systematic study of the effect of CH_4 and O_2 concentrations on the methanotrohic community structure in soil is lacking. Therefore, we investigated the effect of different O_2

and CH_4 mixing ratios upon the methanotrophic population structure and activity in rice field soil using molecular techniques targeting genes of ribosomal RNA and of methanotrophic key enzymes and by analysis of radiolabeled phospholipid ester-linked fatty-acids (^{14}C-PLFAs).

Materials and Methods

Soil. The paddy soil originated from a rice field in Vercelli, Italy. The soil was collected from the drained field on April 18, 1999 before plowing. The fields had been fertilized with 26 Kg N-ha^{-1} to accelerate straw decomposition 18 days prior to soil collection. The soil was air-dried and stored at room temperature. The soil had a maximum water-holding-capacity (WHC) corresponding to a gravimetric water content of 47 ± 1% (n=5). In the experiments described below the soil was moistened with Ammonium-Mineral-Salt-Medium (AMS-Medium) until the gravimetric water content was 20% (wt/wt), which corresponded to 43 % of WHC. The AMS-Medium pH 6.8 contained 9.4 mM NH_4Cl and 3.7 mM KH_2PO_4 and was used to prevent N-limitation and to enhance methanotrophic growth in the soil (Green, 1992). The moist soil was sieved with a 2-mm sieve in order to ensure homogeneity.

Continuos flow set-up. To keep the O_2 and CH_4 mixing ratio constant during the incubation a continuos flow set-up was used. The set-up consisted of a 27-l gas-tight flexible bag functioning as a gas reservoir (Plastigas-Beutel 27l, Roth, Karlsruhe, Germany) and a 1.1-l glass-flask, equipped with a gas inlet and outlet and closed with a latex stopper. The flask and the gas reservoir were connected with isoversinic tubing and equipped with a membrane pump (FM1101 F, Fuergut, Aichstetten, Germany) and a gas flowmeter (0.2-2.0 l min^{-1}, Fuergut, Aichstetten, Germany). The set-ups were checked for gas leakage.

Rice field soil (70 gfw) was weighed into the glass flasks. The gas to soil volume ratio (approximately 400:1) ensured a constant CH_4:O_2 mixing ratio in the continuos flow set-up (Table1). The soil and flasks of set-ups high CH_4/low O_2 (HMLO) and low CH_4 /low O_2 (LMLO) incubated under 1% O_2, were flushed with N_2 before opening the gas flow to remove all atmospheric O_2 (Table1). Gas-flow was adjusted to 0.2 l min^{-1} exchanging the atmosphere in the flasks constantly. Sterile water (10 ml) was injected into the gas-reservoir to adjust for humidity and prevent drying of the soil. The experiments were conducted at 25°C. The gas mixtures in the different incubation set-ups were adjusted as described in Table 1. The atmospheric mixing ratio of CO_2 (300 ppmv) was added to the incubation set-ups. The CH_4 and O_2 mixing ratios were measured periodically by GC and the CH_4 / O_2 mixture was adjusted when necessary.

Rice soil samples were taken periodically from each incubation set-up and stored at –20°C.

CH_4 oxidation. CH_4 oxidation rates were determined from first-order decreases in headspace CH_4. The CH_4 oxidation process was measured and followed in separate closed serum bottles. Sieved rice field soil (4 gfw) was incubated in 120-ml bottles. The gas mixture in each of the bottles was adjusted to approximately the same CH_4 /O_2 mixing ratio as in the corresponding continuos flow set-ups (Table. 1). Three bottles were incubated in parallel at each gas mixture. Headspace CH_4 and O_2 (0.25 ml) was periodically sampled with a pressure lock gas-syringe (Baton-Rouge,) and measured by gas-chromatography (GC). A GC (SRI 5610a, Las Vegas, Nev.) equipped with a thermal-conductivity-detector (TCD) and flame-ionization-detector (FID) mounted in series and operating on synthetic air (20,5% O_2 in N_2) and H_2 was used. Headspace CH_4 was detected by the FID, while O_2 was detected by the TCD. The oven at an oven temperature of 60°C. Gases were separated on a 3 m molsieve-column (5Å), mesh 80/100, Ø 1/8" with He as the carrier-gas.

Generation of $^{14}CH_4$. The acetoclastic methanogenic archeum *Methanosaeta* was incubated in a 120-ml serum flask at 30°C with labeled [2-^{14}C]acetate (37 MBq) as described by Scholten et al. (2000). After less than 8 days was all labeled acetate quantitatively converted to $^{14}CH_4$. Headspace CH_4 had an activity of up to 18 µCi ml^{-1}.

Labeling of methanotrophs with $^{14}CH_4$. After 9, 19 and 33 days (day 33 set up low CH_4/high O_2 (LMHO), only) two parallel soil samples (4 gfw) were taken from each continuos flow set-up and transferred to 60 ml serum bottles. The CH_4, O_2 mixture was adjusted to the mixture in the continuos flow set-up and the headspace was spiked with 6 µCi $^{14}CH_4$. The serum bottles were connected via silicon tubing to a concentrated (5 M) NaOH solution to trap $^{14}CO_2$ produced in the labeling experiment. The labeling was completed after 3-5 days when at least 5 µCi $^{14}CH_4$ had been consumed. The labeled soil was stored at -20°C.

PLFA analysis. Radiolabeled and non-labeled microbial PLFAs were extracted from soil samples and analyzed as described previously (Roslev et al., 1998; Roslev and Iversen, 1999). Extraction and analysis was performed for duplicate soil samples from each incubation.

DNA extraction. DNA extraction from rice field soil was modified after Moré *et al*. (1994). Soil from the frozen samples of the continuos flow set-ups (0.6 gfw soil) was transferred into 2-ml screw cap tubes. Approximately 1 g of sterilized (170°C for 4 h) zirconia/silica beads (0.1 mm diameter; Biospec products Inc., Bartlesville, Ok, USA), 800 µl Na-phosphate buffer (120 mM, pH 8), and 260 µl SDS-solution (10% SDS, 0.5 M Tris/HCl, pH 8.0, 0.1 M NaCl) were added to the soil, and resuspended homogeneously by vorte-

xing. Cells were lysed for 45 s by shaking in a cell disrupter (FP120 FastPrep, Savant instruments Inc., Farmingdale, NY, USA) at a setting of 6.5 m s^{-1}. After centrifugation (3 min at 12,000 × g) the supernatant was collected, and the soil-beads mixture was extracted a second time by resuspension in 700 µl phosphate buffer. Proteins and debris were precipitated from the supernatant by adding 0.4 volumes of 7.5 M ammonium acetate, followed by incubation on ice for 5 min. After centrifugation at 12,000 × g for 3 min nucleic acids were precipitated by addition of 0.7 volumes of isopropanol, followed by centrifugation at 12,000 × g and 4°C for 45 min. Subsequently, the DNA pellet was washed with 70% ethanol at 4°C, and dried under vacuum. Finally, DNA was resuspended in 150 µl Tris-EDTA buffer (10 mM Tris-base, 1 mM EDTA, pH 8).

Humic acids were removed with acid-washed polyvinyl-polypyrrolidone (PVPP) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in Spin columns (BioRad, Munich, Germany) modified after Holben et al. (1988). The spin columns were filled with 2 ml PVPP, which had been equilibrated and suspended in Tris-EDTA pH 8. The PVPP columns were packed and dried by centrifugation (375 x g, 1 min) just prior to loading. About 150 µl of the humic acid-containing soil DNA extract was loaded onto the column and centrifuged. The purified DNA solution was readily amplifiable by PCR.

Mixing ratio and purity of the DNA solutions were determined by absorption at 260 nm and 280 nm after 1:10 dilution in H_2O using a GeneQuant spectrophotometer (Pharmacia Biotech, Upsala, Sweden). For PCR amplification, DNA aliquots at a standardized DNA mixing ratio of 5 ng μl^{-1} were used.

PCR amplification. For PCR amplification we used three SSU rRNA-based primer sets, i.e. an ”universal” primer set targeting all life and the primer sets "MB10γ" and "MB9α", targeting methylotrophic γ- and α-*Proteobacteria*, respectively (Henckel et al., 1999). Further, two functional primer sets were used, i.e. "pmoA" and "mxaF", targeting key-enzymes present in all methanotrophs the particulate methane monooxygenase (pMMO) and the methanol dehydrogenase (MDH), respectively (McDonald &Murrell, 1997 a & b). All used primer pairs had a GC-clamp attached to the 5´end of one primer for subsequent DGGE analysis (Henckel et al., 1999).

PCR buffer (20 mM Tris-HCl, pH 8.3, 50 mM KCl), 1 U *AmpliTaq* DNA polymerase (Perkin Elmer Applied Biosystems, Weiterstadt, Germany), 0.5 µM of each primer, 100 µM of each deoxynucleoside triphosphate (Amersham Life Science, Braunschweig, Germany), and 1 µl of template DNA were added to a total reaction volume of 50 µl at 4°C. In PCR amplifications with primer sets MB10γ, MB9α and pmoA the MasterAmp 2×PCR premix F was used. With primer-set mxaF the MasterAmp 2×PCR premix E was used. The 2×PCR premixes contained 100 mM Tris-HCl (pH 8.3), 100 mM KCl, and 400 µM of each deoxy-

nucleoside triphosphate and 4x PCR-enhancer, the mixing ratios of $MgCl_2$ varied with 5 mM and 7 mM $MgCl_2$ for Premix E and F, respectively (Epicentre Technologies, Madison, WI, USA). Amplifications were started by placing cooled (+4°C) PCR tubes immediately into the preheated (94°C) thermal block of a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). The thermal cycling profiles consisted of touchdown programs with an initial denaturation of 3 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at the annealing temperature, and 45 s at 72°C, and 5 min at 72°C for the last cycle. The annealing temperature decreased from 62°C to 55°C and from 60°C to 50°C in 0.5°C steps for the pmoA and Universal primer sets, respectively. The thermal cycling profile for primer sets MB10γ, MB9α and mxaF consisted of an initial denaturation of 3 min at 94ºC, of 30 s at 94ºC, 30 s at the annealing temperature of 60°C for MB10γ and MB9α, and 55°C for mxaF, and 1 min at 72ºC. The number of cycles was 29 for MB10γ, and 31 for MB9α and mxaF, the last cycling was followed by 5 min at 72ºC for final elongation.

Aliquots (5 µl) of PCR products were analyzed by electrophoresis on 3% agarose gels, stained with ethidium bromide, and quantified densitometrically. Gels were destained in water for 30 min. For calibration the Smart-Ladder DNA mass and size ruler (Eurogentec, Seraing, Belgium) was used (calibration coefficient of all analyses $r > 0.9$). Gels were photographed with an imaging system (MWG Biotech, Germany), and DNA bands were analyzed with the RFLP-scan software (CSP Inc., Billerica, Belgium).

DGGE. DGGE was carried out as previously described (Henckel et al., 1999). PCR products were separated using the DCode System (Bio-Rad, Munich, Germany) on 1-mm thick polyacrylamide gels (6.5% w/v acrylamide:bis acrylamide (37.5:1); Bio-Rad, Munich, Germany) prepared with and electrophoresed in 0.5 × TAE, pH 7.4 (0.04 M Tris-base, 0.02 M sodium-acetate, 1 mM EDTA) at 60°C, and constant voltage. A denaturing gradient of 35-80% for pmoA, 35-70% for universal and mxaF, and 40-70% for MB10γ and MB9α PCR products was used. A denaturing gradient of 80% (vol/vol) denaturant corresponded to 6.5% acrylamide, 5.6 M urea and 32% deionized formamide. Gels were poured onto GelBond PAG film (FMC Bioproducts, Rockland, ME, USA) to avoid gel distortion. Gels were stained with 1:50,000 (vol/vol) SYBR-Green I (Biozym, Hessisch-Oldendorf, Germany) for 30 min, and scanned with a Storm 860 phosphor imager (Molecular Dynamics, Sunnyvale, CA, USA). The scanned DGGE gels were, as a whole, digitally enhanced with ImageQuant 5.0 (Molecular Dynamics, Sunnyvale, CA, USA) or Adobe-Photoshop 5.0 (Adobe Systems Incorporated).

For further analysis, DGGE bands were visualized in the SYBR Green I-stained gels with blue light ($\lambda > 400$ nm) using a Dark Reader transilluminator (Clare Chemical Re-

search, Ross on Wye, UK). Individual DGGE bands were then excised, reamplified, and reanalyzed by DGGE to verify band purity (Henckel et al., 1999).

Sequencing of DGGE bands. Reamplified PCR products of excised DGGE bands were purified using the EasyPure DNA purification kit (Biozym, Hessisch-Oldendorf, Germany). Mixing ratio and purity of PCR products were determined by absorption at 260 nm and 280 nm of a 1:20 dilution in H_2O with a GeneQuant spectrophotometer (Pharmacia Biotech, Upsala, Sweden). Sequencing reactions were performed using the ABI Dye-terminator cycle sequencing kit (Perkin Elmer Applied Biosystems,) as specified by the manufacturer. Cycle sequencing products were purified from excess dye terminators and primers using Microspin G-50 columns (Pharmacia Biotech, Freiburg, Germany), and analyzed with an ABI 373 DNA sequencer (Perkin Elmer Applied Biosystems).

Sequences were analyzed using the Lasergene software package (DNASTAR, Madison, WI, USA). Nucleotide and inferred amino acid sequences of the gene fragments of *pmo*A and *mxa*F were manually aligned with sequences retrieved from the GenBank database. SSU rDNA sequences were aligned and phylogenetically placed with the ARB software package (Strunk and Ludwig, 1996). The partial SSU rDNA sequences were added using the maximum parismony method to a validated and optimized tree of about 5000 SSU rDNA sequences, while keeping the overall topology constant (Ludwig et al., 1998). Phylogenetic trees were constructed by using distance matrix and maximum parsimony methods supplied by the ARB software package (http://www.biol.chemie.tu-muenchen.de/pub/ARB/). On the nucleic acid level, evolutionary distances between pairs of sequences were calculated by using the Jukes-Cantor, and Felsenstein equations (Jukes and Cantor, 1969; Felsenstein, 1993) implemented in the ARB package.

Nucleotide accession numbers. The sequences of *pmo*A, *mxa*F and SSU gene fragments of excised DGGE bands have been deposited in GenBank under accession numbers XXXXX, respectively.

Table 1. Incubation set-ups were labeled according to the CH_4 /O_2 concentrations, i.e. H, high; L, low; M, methane; O, oxygen. CH_4 and O_2 mixing ratios are given as mean ± standard deviation (SD) mixing ratio during incubation of the continuos flow set-ups (n=30). Values in () show initial gas concentration ± SD of the batch set-ups in which CH_4 depletion was measured (n=3). CH_4 oxidation rates were calculated from linear regression of CH_4 depletion curves (confidence limit 5%), error given as standard error (SE) (n=3). All incubations were contained ambient CO_2 (300 ppmv).

incubation set-up	**Gas mixing ratios**		**CH_4 oxidation rate [nmol h^{-1} gdw^{-1}]**		**factor of oxidation rate induction**
	CH_4 [ppmv] ± SD	**O_2 [%] ± SD**	initial ± SE	induced ± SE	
HMLO	12,596 ± 2906 (19,222 ± 312)	0.6 ± 0.2 (0.5 ± 0.2)	180 ± 43	3562 ± 113	20
LMHO	781 ± 151 (1,101 ± 32)	20.0 ± 0.4 (20.4 ± 0.3)	4 ± 1	46 ± 1	10
HMHO	12,418 ± 1,397 (18,897 ± 164)	19.2 ± 0.8 (19.3 ± 1.2)	141 ± 80	5184 ± 146	37
LMLO	800 ± 200 (1,070 ± 15)	1.1 ± 0.3 (0.8 ± 0.2)	8 ± 2	61 ± 3	8
ambient air	ambient air (2 ± 1)	ambient air (20.5)	0	0	0

Results

Rice field soil at 43% of WHC was incubated under high and low CH_4 and high and low O_2 in 4 possible variations, and furthermore under ambient air (Table 1). Soil samples for molecular analyses were taken from the continuos flow-set-ups, in which CH_4 and O_2 mixing ratios were kept constant (Table 1). The CH_4 oxidation process was followed in separate closed batch incubations with initial conditions as indicated in Table 1.

CH_4 oxidation in batch incubations. When the rice field soil was incubated in the presence of high CH_4 and low O_2 (HMLO) or high O_2 (HMHO), CH_4 oxidation started after approximately one day at an initial rate of 141 to 180 nmol $h^{-1}gfw^{-1}$ (Fig. 1A). On day 5-6, CH_4 oxidation activity was enhanced to a rate 20-fold (HMLO) to 37-fold (HMHO) (Table 1). In the batch HMLO and HMHO incubations, CH_4 was completely consumed after 8-9 days.

At low CH_4 and high O_2 mixing ratios (LMHO), CH_4 was oxidized at a very low rate of 4 ±1 nmol $h^{-1}gfw^{-1}$ (Fig. 1B). After 19 days of incubation the CH_4 oxidation rate increased 10-fold (Table 1). Methane consumption in the batch incubations ceased at 30 ppmv CH_4 after 42 days.

When rice field soil was incubated in the presence of low CH_4 and low O_2 (LMLO), the CH_4 oxidation rates of 8 ± 2 nmol $h^{-1}gfw^{-1}$ increased already after 7 days 8-fold (Fig. 1B; Table 1), and CH_4 was depleted to 20 ppmv CH_4 after about 20 days (Fig. 1B).

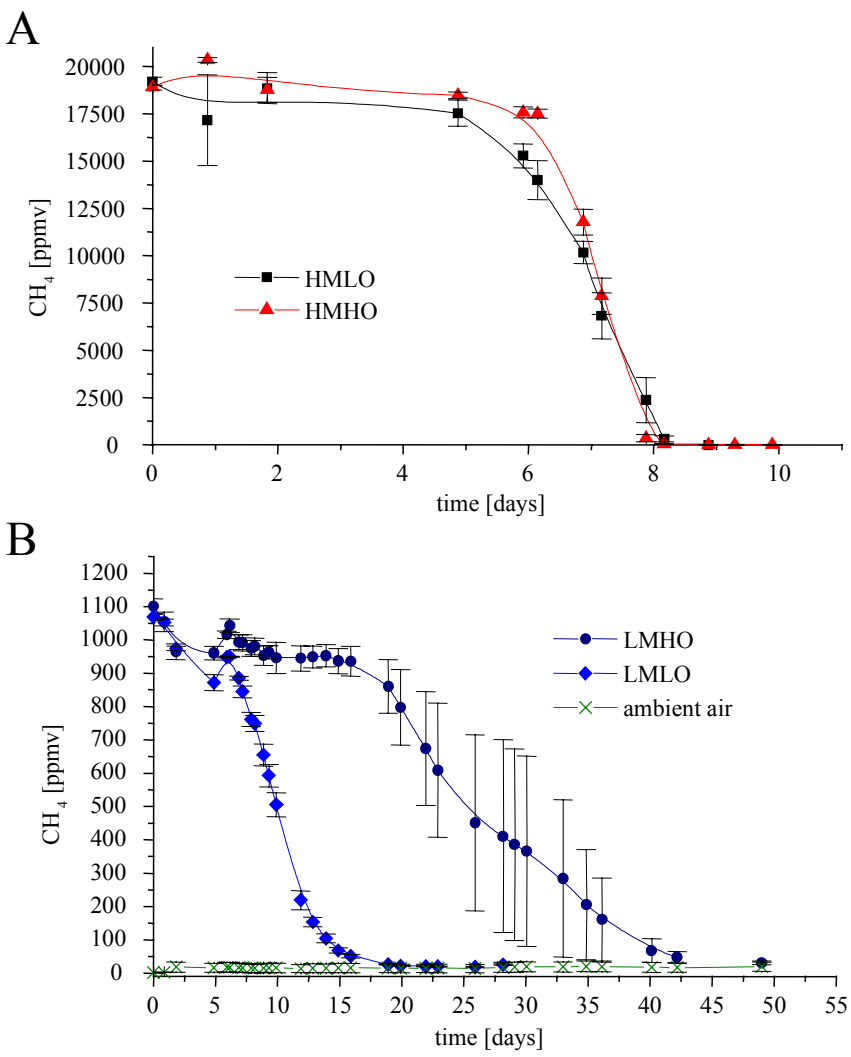

Figure 1. Oxidation of CH_4 in closed serum bottles at (A) high CH_4 with high (HMHO) and low (HMLO) O_2; and (B) at low CH_4 with high (LMHO) and low (LMLO) O_2 and at ambient CH_4 and O_2 (CH_4 and O_2 mixing ratios see Table 1); means ± standard error of n=3.

PLFA analysis. Soil samples were taken from incubation set-ups HMLO, HMHO, LMHO and LMLO after 9, 19 and 33 days and incubated in closed serum flasks with $^{14}CH_4$ to label the PLFAs of the active methanotrophs. ^{14}C-PLFA fingerprints showed that after 9 days, 70% of the $^{14}CH_4$ assimilated into radiolabelled PLFAs in HMLO, and >80% in LMHO and LMLO was incorporated into PLFA fraction 5 (Fig. 2). Incorporation of $^{14}CH_4$ into PLFAs fraction 5 (e.g., 16:1 and 16:0 fatty acids) indicate CH_4 oxidation by mainly type I methanotrophs, whereas ^{14}C-PLFAs in fraction 11 (e.g., 18:1 and 18:0 fatty acids) indicate CH_4 oxidation by mainly type II methanotrophs. After 19 days of incubation, the distribution of labeled PLFA had not changed in incubations under low CH_4 (LMHO and LMLO) clearly indicating that type I methanotrophs continued to dominate the CH_4 metabolism. In con-

contrast, contribution by type II methanotrophs had increased after 19 days to about 50% in incubations with high CH_4 (HMLO and HMHO), as indicated by recovery of approximately equal amounts of radioactivity in PLFA fractions 5 and 11 (Fig. 2).

Because of the long lag phase in LMHO (Fig. 1), a third labeling experiment was conducted after 33 days of incubation. However, type I methanotrophs remained the dominant methane oxidizers with approximately 90% of ^{14}C-PLFAs recovered in PLFA fraction 5 (data not shown).

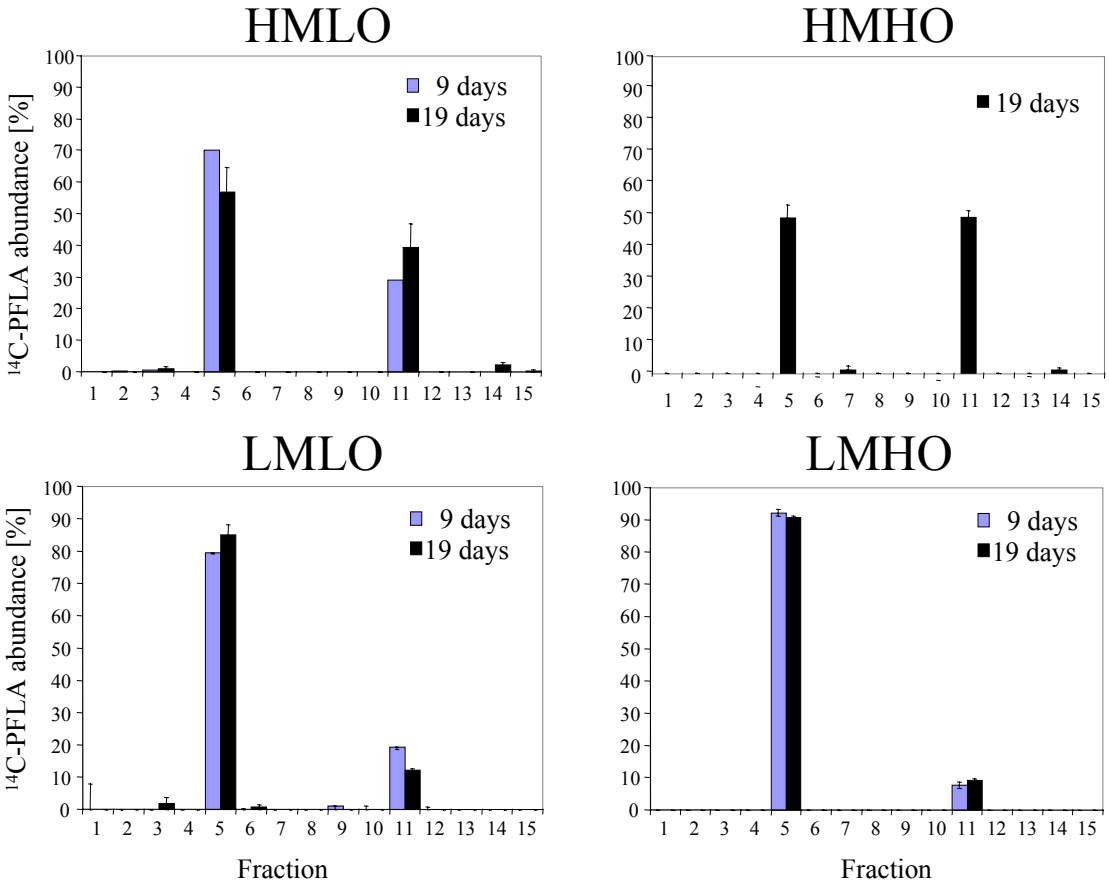

Figure 2. ^{14}C-PLFA fingerprints of methane-oxidizing bacteria from rice field soil incubated under a continuos flow of different CH_4/O_2 atmospheres (designatins see Table 1). The graphs show the percentage of radioactivity detected in each PLFA-fraction. $^{14}CH_4$ incorporation into PLFAs that elute in fraction 5 indicate CH_4 oxidation dominated by type I methanotrophs, whereas fraction 11 indicates CH4 oxidation dominated by type II methanotrophs.

The abundance of methanotrophic bacteria in the rice soil was determined by using the PLFAs 16:1ω8c and 18:1ω8c as biomarkers for type I and II methanotrophs, respectively (Fig. 3). High methane (HMLO and HMHO) resulted in growth of both type I and II methanotrophs as indicated by a 9-39 times increase in the abundance of methanotrophic PLFAs after 9-19 days of incubation, compared to the abundance of PLFAs in LMHO after 9 days (Fig. 3). Low methane mixing ratios (LMHO and LMLO) resulted also in growth of

type I and II methanotrophs with a 4-9 fold increase in total abundance of methanotroph specific PLFAs, compared to the abundance of PLFAs in LMHO after 9 days. We assumed that the PLFA abundance in LMHO represented the initial level in the soil. The type II-specific PLFA 18:1ω8c was the dominant methanotrophic biomarker at high CH_4 whereas the type I-specific PLFA 16:1ω8c was relatively more abundant (2-fold) at low CH_4 (Fig. 3).

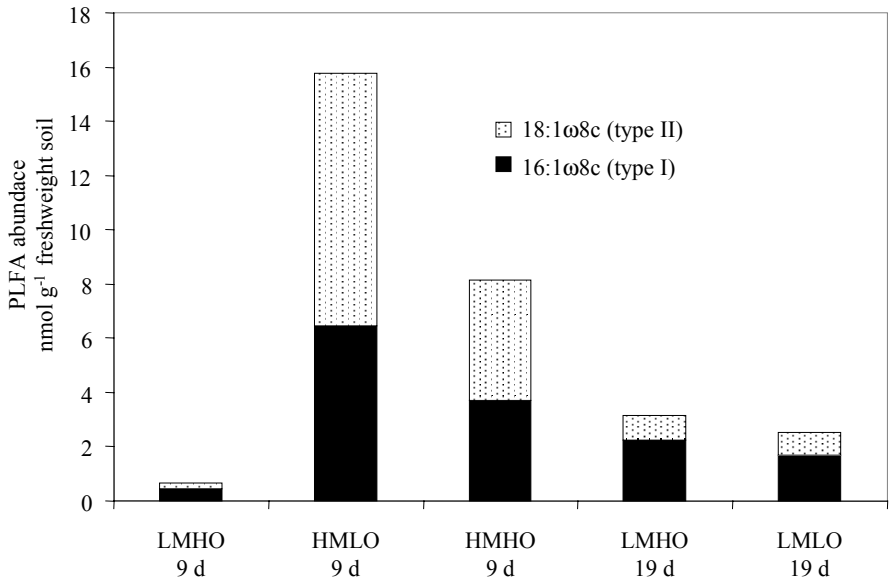

Figure 3. Total abundance of type-I-specific 16:1ω8c and type-II-specific 18:1ω8c PLFAs in soil samples taken from the continuos-flow set-ups after induction of CH_4 oxidation, i.e. LMHO 9 days, HMLO 9 days, HMHO 9 days, LMHO, 33 days, LMLO 19 days.

PCR-DGGE. Soil samples were taken from the continuos flow set-ups at the times indicated, and DNA was extracted and used for PCR amplification. PCR products of the predicted sizes were obtained by amplification with the functional primer sets pmoA and mxaF, and the 16S rDNA primer sets MB9α, MB10γ and UNI, using template DNA extracted from soil samples of the HMLO, LMHO, HMHO, LMLO, and ambient incubations at different time points. The primer sets UNI, MB9α and MB10γ, targeting the 16S rDNA, amplified PCR products at similar concentration yield from all samples, indicating that the template DNA was readily amplifiable and not subjected to biases caused by humic acids or other PCR-inhibiting substances.

Whereas amplification with primer set mxaF resulted in high PCR product yields from template DNA retrieved from soil incubated under high CH_4 (HMLO, HMHO), only low or no PCR products at all were obtained from template DNA from soil under low CH_4 (LMHO, LMLO, ambient). The *mxa*F product yield did not increase at higher template concentration or higher number of PCR-cycles.

SSU rDNA DGGE community pattern. The DGGE analysis of PCR products amplified with the primer set UNI showed similar banding patterns for all of the 5 different soil incubation set-ups and the patterns were uniform with time (Fig. 4), with one exception. One new DGGE band appeared and increased in intensity in HMHO and HMLO after day 5. This DGGE band became most intense in HMLO (Fig. 4), was also present in HMHO (Fig. 4), but. did not appear in LMLO (Fig. 4), LMHO (data not shown) and ambient) (data not shown).

Sequence analysis revealed that the DGGE bands MO1 and MO2 each had identical sequences irrespectively of whether they were retrieved from different incubations or at different times (Fig. 5). The bands MO1 and MO3, which were present in all incubations at all times, clustered within the *Cytophaga-Flavobacterium* (CFB) phylum. MO3 grouped within the gram-positive *Bacteria* with high G+C (Fig. 5). The DGGE band MO1 had an almost identical sequence as a DGGE band earlier detected in Vercelli rice field soil (Henckel et al., 1999). However, band MO2, which appeared in HMHO and HMLO incubations, belonged to the α-*Proteobacteria*, closely related to the type II methanotrophs *Methylocystis spp*. and *Methylosinus spp*. (Fig. 5).

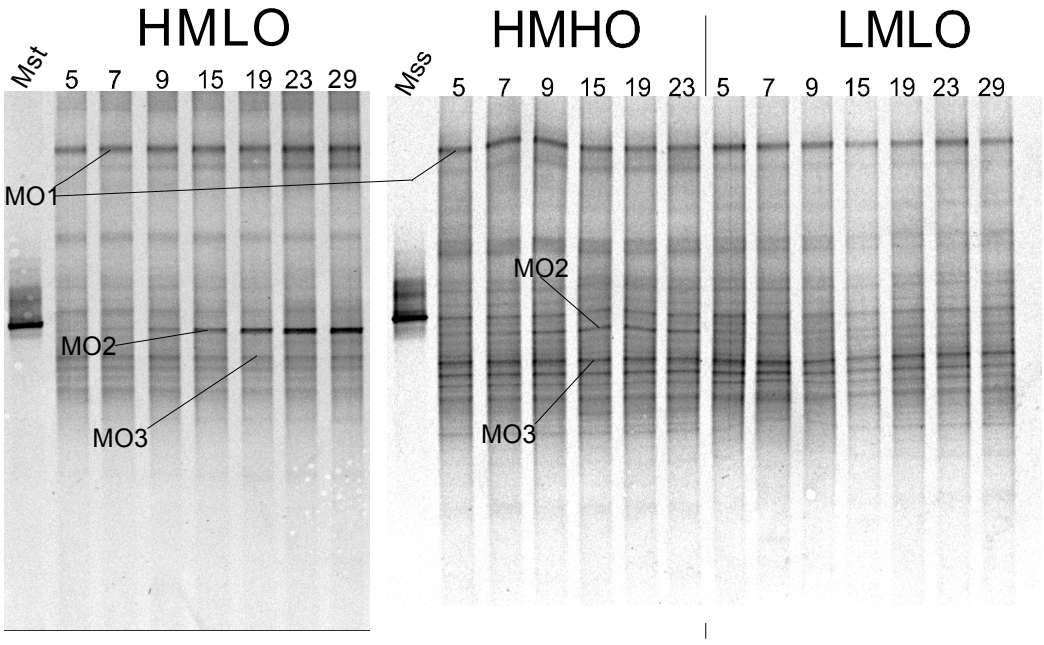

Figure 4. DGGE banding pattern obtained after amplifying DNA extracted from rice field soil incubated under a continuos flow of different CH_4/O_2 atmospheres (designation see Table 1) with the universal SSU rDNA primer set (UNI). Soil samples for DNA extraction were taken at the times indicated [days]. Bands of marked position were sequenced. Mst, *Methylosinus trichosporium*; Mss, *Methylosinus sporium*.

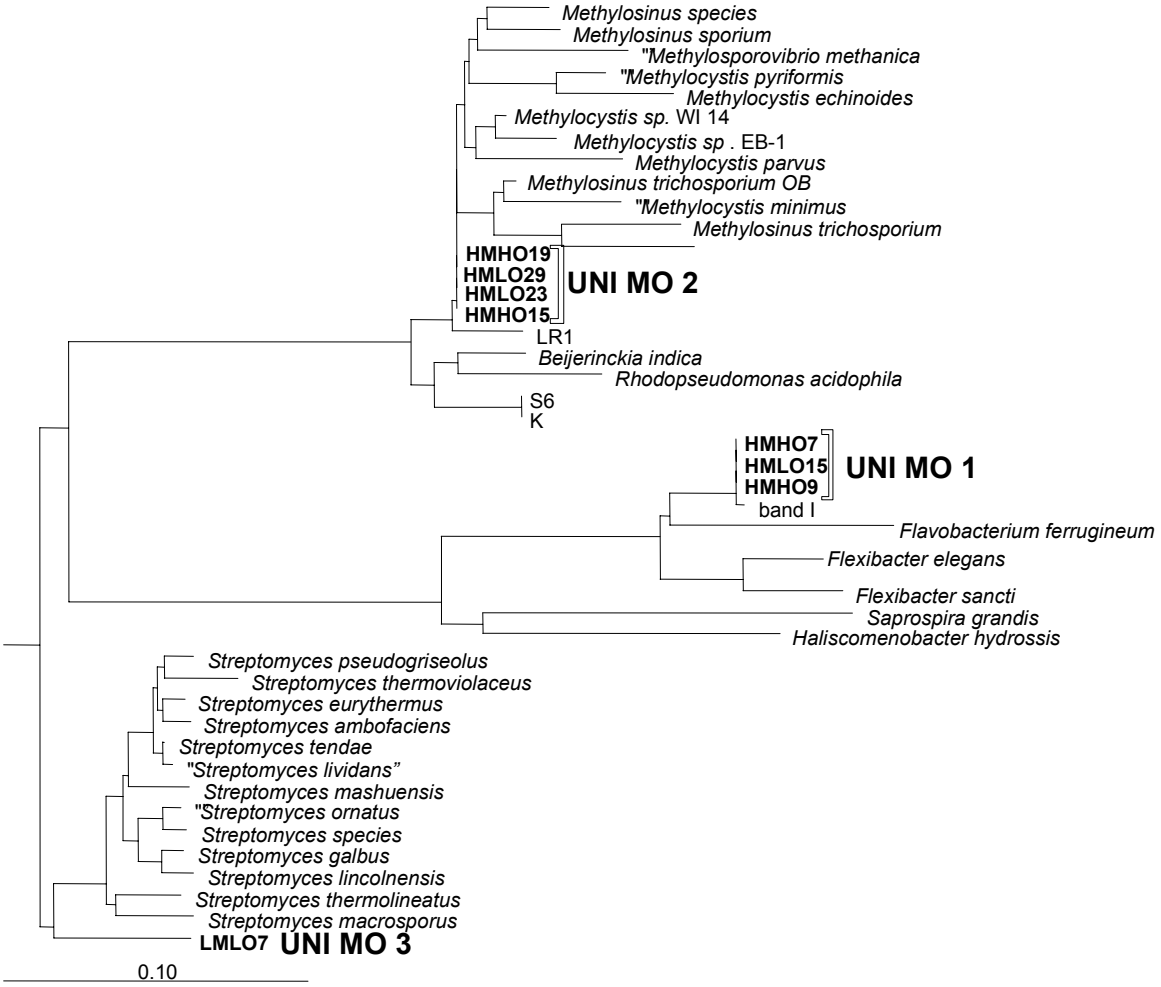

Figure 5. Phylogenetic tree constructed with partial SSU rDNA sequences retrieved from UNI DGGE bands (see Fig. 2), showing the relationship of the marked DGGE bands to related members of the domain *Bacteria*. The partial sequences were added by maximum parsimony to a verified tree without changing the tree topology. The scale bar represents estimated number of base changes per nucleotide sequence position. Individual DGGE band sequences are labeled according to incubation set-up (HMLO, HMHO, LMHO, LMLO) and the day the sample was taken. UNI MO 1 to UNI MO 3, position of marked universal DGGE band; LR1, high affinity type II methanotroph; K, S6 acidophilic methanotroph; band I, UNI DGGE band sequence retrieved from rice field soil.

MB9α rDNA DGGE community pattern. The DGGE analysis of PCR products amplified with the MB9α primer set showed an identical banding pattern with time in incubations HMHO (Fig. 6), HMLO (data not shown), LMHO (data not shown), LMLO (Fig. 6) and ambient (Fig. 6).

Sequence analysis revealed that all bands grouped within the α-*Proteobacteria* (Fig. 7). The most pronounced band MO2**,** present in all incubations at all times, grouped closest to sequence RRII5 retrieved from the rice rhizosphere (Bodelier et al., 2000) and next closest to the type II methanotrophs *Methylosinus spp*./ *Methylocystis spp*. Sequences of bands MO1and MO3, on the other hand, were not closely related to methanotrophs. Sequence of MO3 was closely related to sequences RRII7 and RRII6 from the rice rhizosphere, and to K20-80, a clone detected in metal-contaminated soil (Fig. 7). Sequence of MO1

was closely related to the MB9α clone sequences labeled c, ox or RRII, which had earlier been retrieved from rice field soil or the rice rhizosphere (Fig. 7). Sequence of band MO3 grouped close to *Rhodopseudomonas* spp, and MO1 close to *Sphingomonas* spp (Fig. 7).

The HMLO incubation showed that some DGGE bands of low electrophoretic mobility changed in intensity with time (data not shown). The electrophoretic mobility of these bands corresponded to MB9α clones ox8, ox11, ox12 and c11 which had earlier been retrieved from Vercelli rice field soil (Henckel et al., 1999) (Fig. 7). These clones were not related to type II methanotrophs and grouped close to *Sphingomonas* spp.

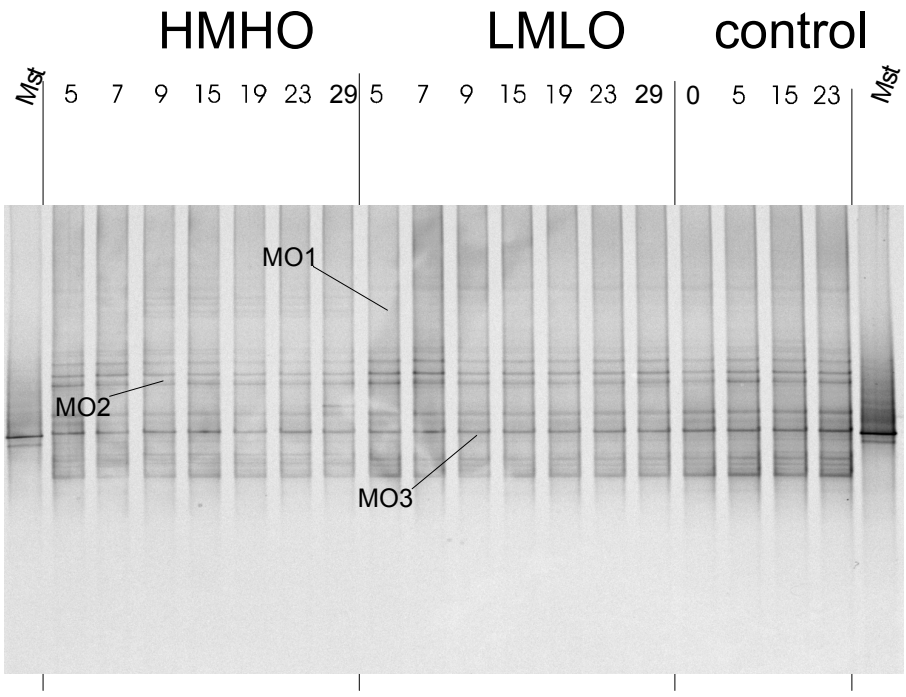

Figure 6. DGGE banding pattern obtained after amplifying DNA extracted from rice field soil incubated under a continuos flow of different CH_4/O_2 atmospheres (designation see Table 1) with the SSU rDNA primer set targeting type II methylotrophs (MB9α) . Soil samples for DNA extraction were taken at the times indicated [days]. Bands of marked position were sequenced. Mst, *Methylosinus trichosporium*.

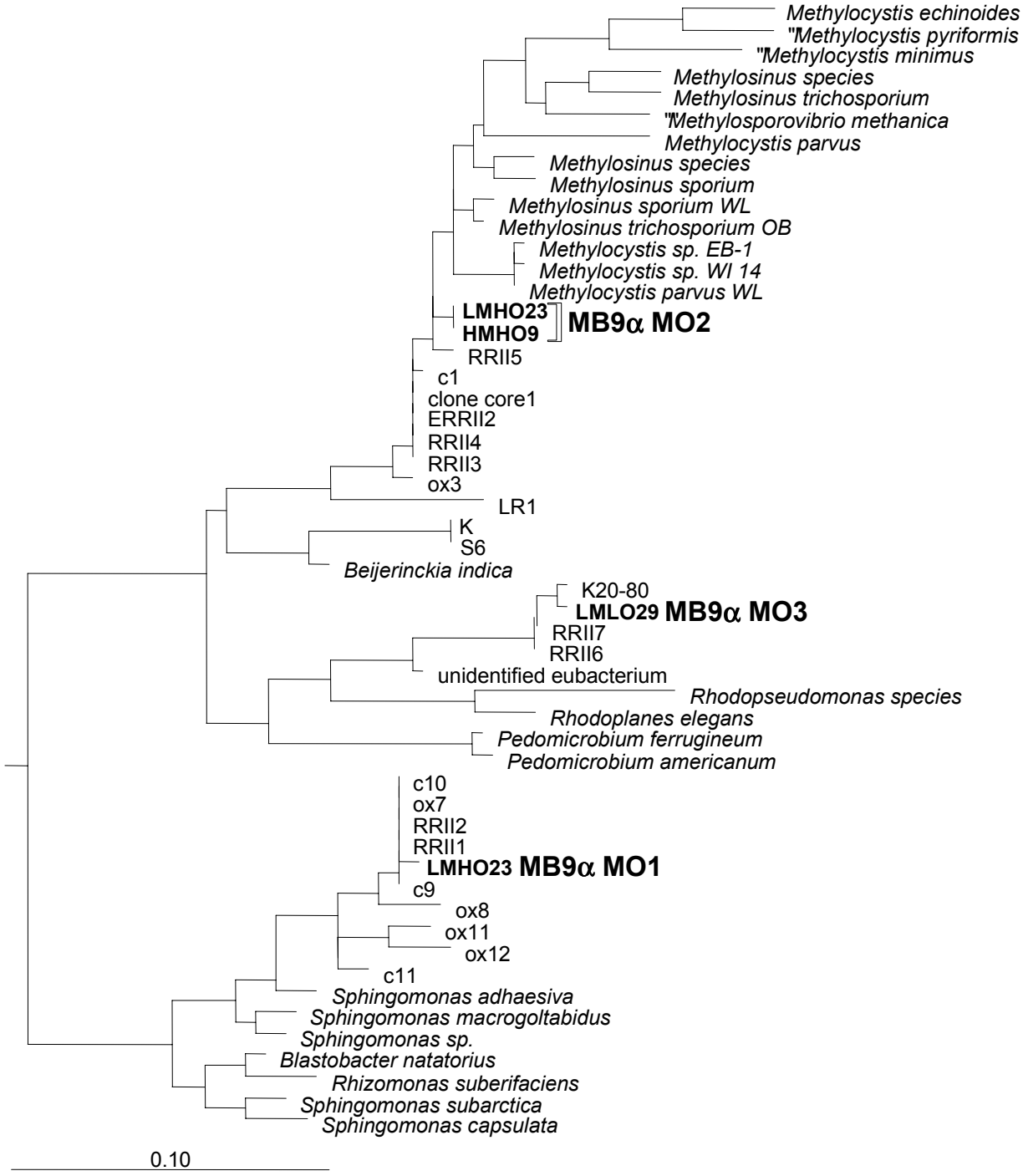

Figure 7. Phylogenetic tree constructed with partial SSU rDNA sequences retrieved from MB9α DGGE bands (see Fig. 4), showing the relationship of the marked DGGE bands to related members of α-*Proteobacteria*. The partial sequences were added by maximum parsimony to a verified tree without changing the tree topology. The scale bar represents estimated number of base changes per nucleotide sequence position. Individual DGGE band sequences are labeled according to incubation set-up (HMLO, HMHO, LMHO, LMLO) and day the sample was taken. MB9α MO 1-3, position of marked DGGE bands; LR1, high affinity type II methanotroph; K, S6 acidophilic methanotroph; ”c”, ”ox”, are clones retrieved from rice field soil incubated without and with CH_4, respectively; ”RRII”, are clones retrieved from rice rhizosphere soil, K20-80, clone retrieved from metal-contaminated soil.

MB10γ rDNA DGGE community pattern. The DGGE analysis of PCR products amplified with the MB10γ primer set showed differences in banding patterns depending on the CH_4 mixing ratio. Incubations HMHO and HMLO showed the same banding pattern dominated by two very intense DGGE bands, MO4 and MO5 (Fig. 8). The ambient, LMHO and LMLO incubations, on the other hand, showed a banding pattern with >10 dominant DGGE-bands, indicating a higher diversity at low CH_4 incubations (Fig. 8). Comparison of the complex banding pattern of the ambient incubation on day 0 to the banding pattern of the HMLO and HMHO incubations shows a reduction of the number of DGGE bands during the first 5 days of incubation, probably indicating a shift of the population structure induced by high CH_4 mixing ratios (Fig. 8).

The overall banding pattern of LMHO and LMLO were similar. In HMLO and HMHO, the most dominant DGGE bands MO4 and MO5 were present from the beginning (day 5). However, in LMHO and LMLO, incubated under low CH_4, these bands started to appear on day 9, and were completely missing in the ambient incubation (Fig. 8).

Sequence analysis revealed that all bands grouped within the γ-*Proteobacteria* (Fig. 9). DGGE bands MO1, MO2, MO3, MO4 and MO5, retrieved from different incubations and time points, all grouped within the type I methanotrophs. Bands MO1 and MO2 were closest related to the MB10γ sequences RRI2 and RRI4 retrieved earlier from rhizospere soil (Bodelier et al., 2000) and to *Methylobacter spp*. Bands MO4 and MO5, most dominant under high CH_4, as well as MO3 were closest related to *Methylomonas* spp. On the other hand, the DGGE bands with higher electrophoretic mobility MO6 and MO7 were not related to known type I methanotrophs, but grouped close to *Xylella* and to sequences BPC023 and BHB6, retrieved form a hydrocarbon seep (AF154087) and from an ammonia biofilter (AF090542, Sakano & Kerkhof, 1998), respectively (Fig. 9).

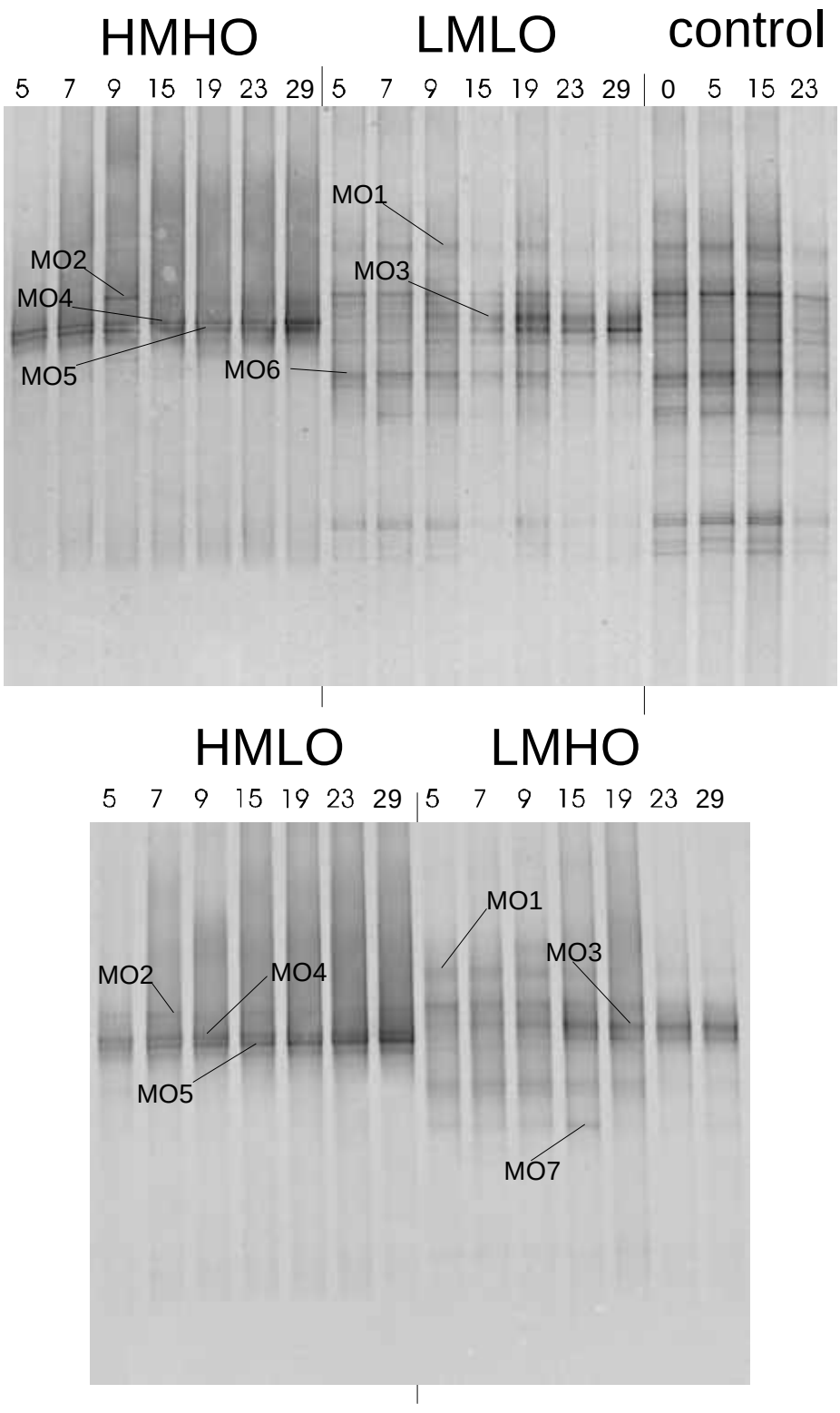

Figure 8. DGGE banding pattern obtained after amplifying DNA extracted from rice field soil incubated under a continuos flow of different CH_4/O_2 atmospheres (designation see Table 1) with the SSU rDNA primer set targeting type I methylotrophs (MB10?). Soil samples for DNA extraction were taken at the times indicated [days]. Bands of marked position were sequenced.

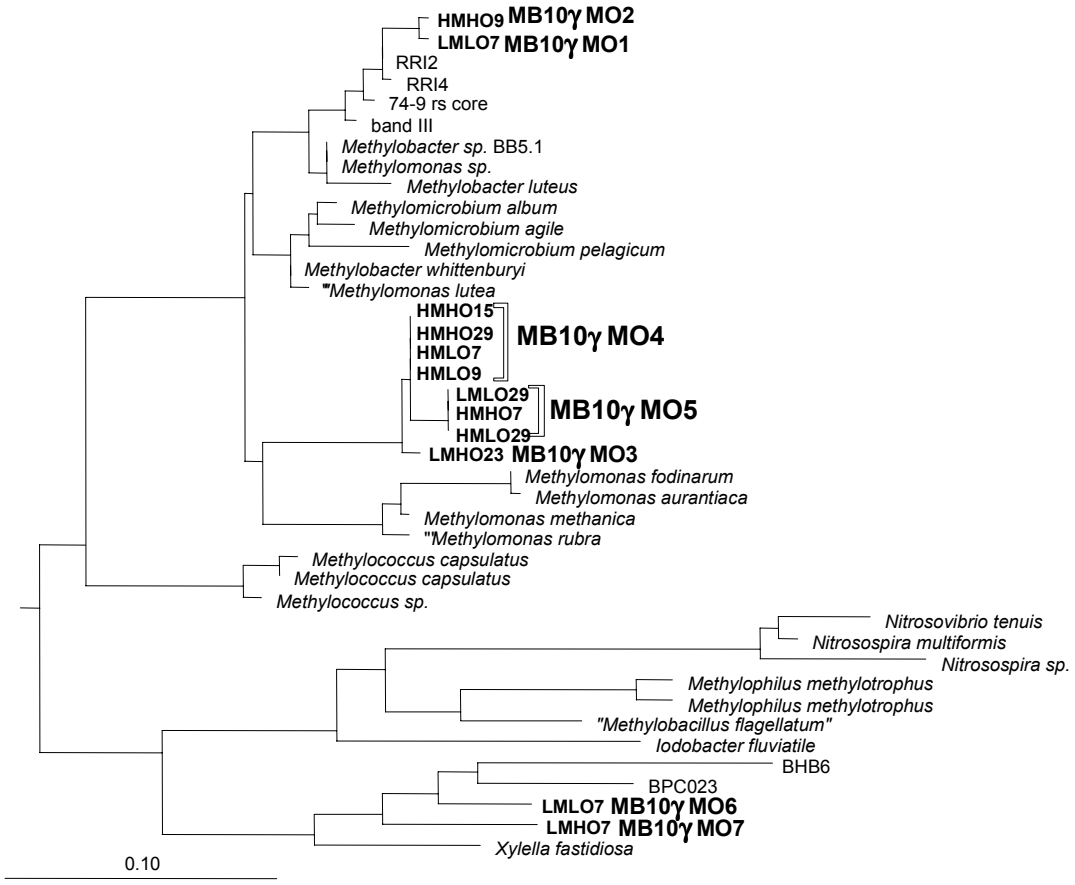

Figure 9. Phylogenetic tree constructed with partial SSU rDNA sequences retrieved from MB10γ DGGE bands (see Fig. 6), showing the relationship of the marked DGGE bands to related members of γ -*Proteobacteria*. The partial sequences were added by maximum parsimony to a verified tree without changing the tree topology. The scale bar represents estimated number of base changes per nucleotide sequence position. Individual DGGE band sequences are labeled according to incubation set-up (HMLO, HMHO, LMHO, LMLO) and day the sample was taken. MB10γ MO 1-7, position of marked DGGE bands; ”RRI”, are clones retrieved from rice rhizosphere soil; band III, 74-9rscore, MB10γ DGGE bands sequence retrieved from rice field soil incubated with CH_4 and from a drained rice soil core, respectively; BHB6, clone retrieved from an ammonia biofilter, BPC023, clone sequence retrieved from a hydrocarbon seep.

***mxa*F DGGE community pattern**. The DGGE analysis of PCR products amplified with the mxaF primer set showed different banding patterns depending on the O_2 and CH_4 mixing ratios (Fig. 10). Incubations LMHO (data not shown), LMLO (Fig. 10) and ambient(data not shown) showed only very faint banding patterns due to low PCR product yield. The *mxa*F gene fragment was only amplified at sufficient PCR product yield in soil incubated under high CH_4 mixing ratios, i.e. HMLO (Fig. 10) and HMHO (Fig. 10).

The banding patterns of HMLO and HMHO showed an increase in the number of bands and band intensity with time (Fig. 10). In HMHO, band number increased from two bands during the first 7 days to three bands after day 9 (Fig. 10). HMLO showed 6 dominant bands that gradually appeared after 7 days of incubation (Fig. 10). HMLO showed a higher number of bands than HMHO indicating a greater diversity (Fig. 10). Since both

HMLO and HMHO were incubated under high CH_4, differences in the community structure must have been caused by the O_2 mixing ratio. In the soil incubated under low CH_4 mixing ratios (LMHO, LMLO), only three very faint bands were visible (Fig. 10).

Analysis of the amino acid sequences derived from the DNA sequences of the DGGE bands revealed that band MO5, present in HMLO and HMHO, grouped within the type II methanotrophs, closest to *mxa*F sequences of band B and con11PP retrieved earlier from Vercelli rice field soil incubated under high CH_4 and from the rice rhizosphere, respectively (Fig. 11). Band MO4, faintly visible in LMLO and LMHO after 19 days, grouped within the same cluster of type II methanotrophs (Fig. 11). Sequence of MO3 detected in HMLO was also closest related to type II methanotrophs (Fig. 11). However, the sequences of bands MO1 and MO2, only present in HMLO, were closest related to type I methanotrophs of the genus *Methylomicrobium*. (Fig. 11). Sequences of MO6 and MO7, detected in all incubations were closest related to methylotrophs of the genus *Hyphomicrobium* (Fig. 11).

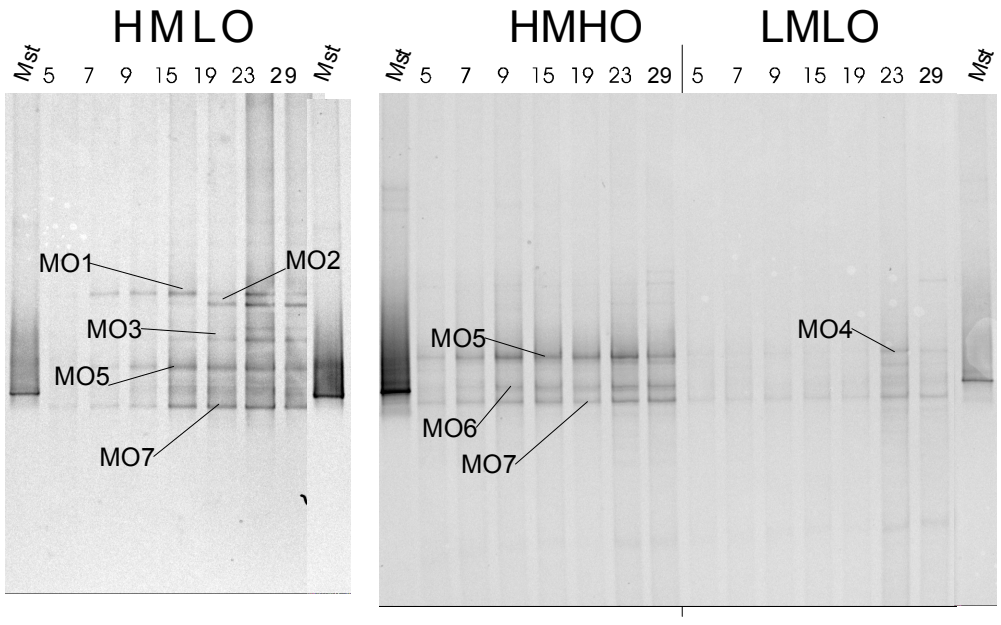

Figure 10. DGGE banding pattern obtained after amplifying DNA extracted from rice field soil incubated under a continuos flow of different CH_4/O_2 atmospheres (designation see Table 1) with the functional primer set targeting the gene of the α-subunit of the MDH present in all Methylotrophs (mxaF). Soil samples for DNA extraction were taken at the times indicated [days]. Bands of marked position were sequenced. Mst, *Methylosinus trichosporium*.

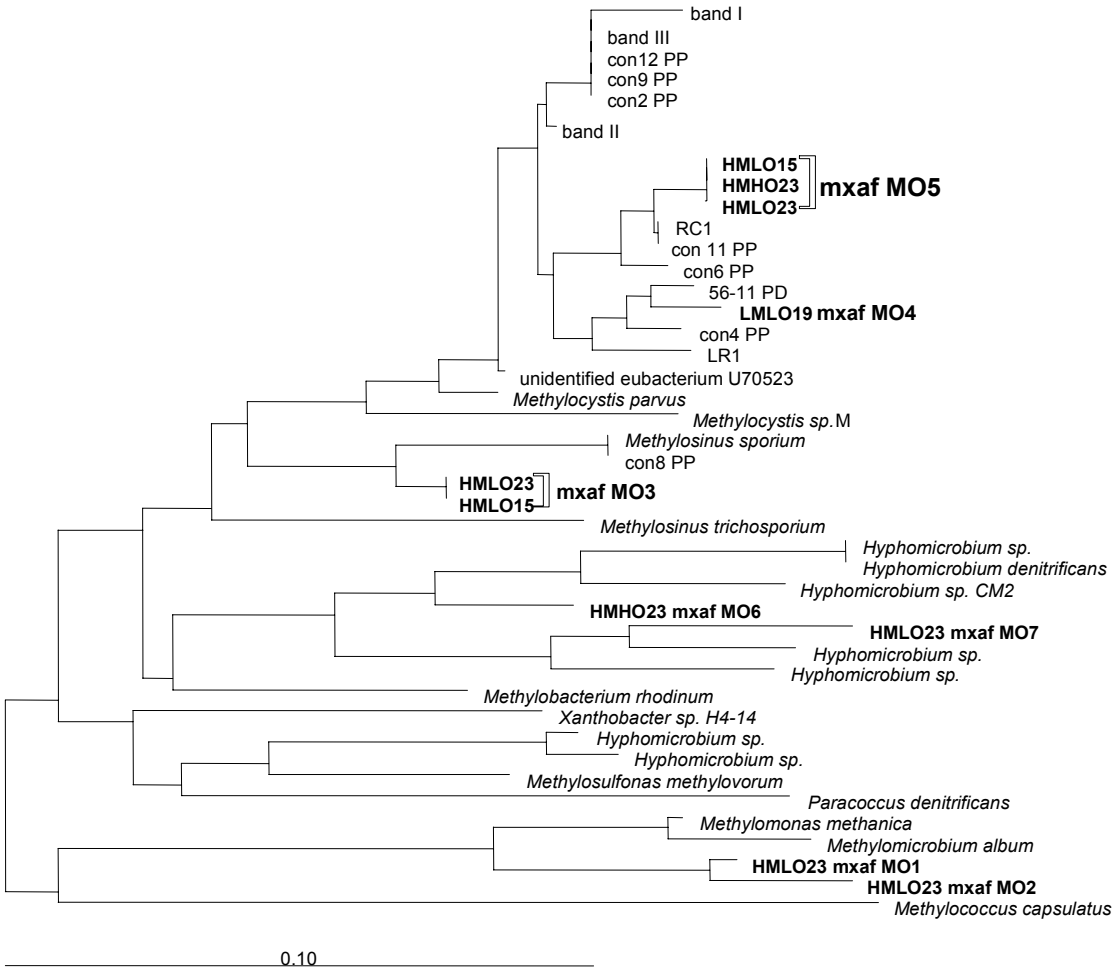

Figure 11. Phylogenetic tree based on the derived amino acid sequences of *mxa*F sequences retrieved from mxaF DGGE bands (see Fig. 8), showing the relationship of the marked DGGE bands to other methylotrophs. The scale bar represents estimated number of changes per amino acid sequence position. Individual DGGE band sequences are labeled according to incubation set-up (HMLO, HMHO, LMHO, LMLO) and day the sample was taken. mxaf MO 1-5, position of marked DGGE bands; "con PP", are mxaf DGGE band sequences retrieved from rice rhizosphere soil (unpublished data); 56-11PD, mxaf DGGE band detected in an enrichment culture from a humisol (unpublished data); band I, II, II, mxaf DGGE band sequences retrieved from rice field soil incubated with CH_4; RC1, mxaf DGGE band sequence retrieved from drained rice field soil; LR1, high affinity type II methanotroph.

***pmo*A DGGE community pattern**. The DGGE analysis of PCR products amplified with the pmoA primer set showed a similar DGGE pattern for all incubations (Fig. 12). The banding pattern for LMHO (data not shown), LMLO (Fig. 12) and ambient (Fig. 12) was identical and did not change with time. In HMHO, on the other hand, band MO3 appeared after 15 days of incubation and increased in intensity (Fig. 12).

Sequence analysis revealed that the sequences of MO1 and MO2 grouped within the β-*Proteobacteria*, closest related to *amo*A sequences retrieved from Vercelli rice soil and to ammonium oxidizers of the genus *Nitrosospira* (Fig. 13). The putative ammonium-oxidizer sequences MO1 and MO2 were also dominant in the ambient incubation, probably stimula-

ted by the added nutrient solution containing NH_4^+. Band MO3 that appeared only in HMHO grouped within the α-*Proteobacteria* closest related to type II methanotrophs of the genus *Methylosinus*.

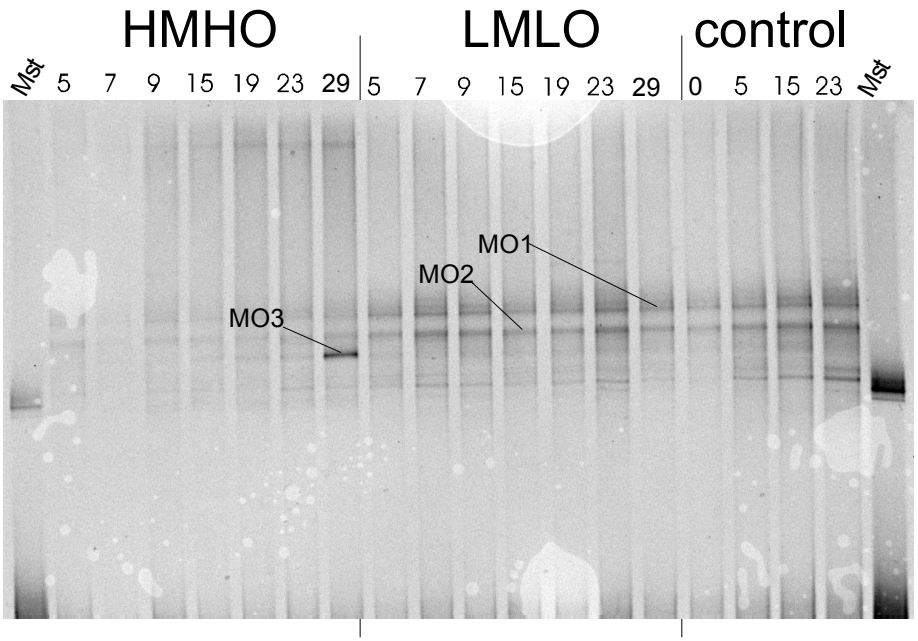

Figure 12. DGGE banding pattern obtained after amplifying DNA extracted from rice field soil incubated under a continuos flow of different CH_4/O_2 atmospheres (designation see Table 1) with the functional primer set targeting the gene of the α-subunit of the pMMO present in all Methanotrophs (pmoA). Soil samples for DNA extraction were taken at the times indicated [days]. Bands of marked position were sequenced. Mst, *Methylosinus trichosporium*.

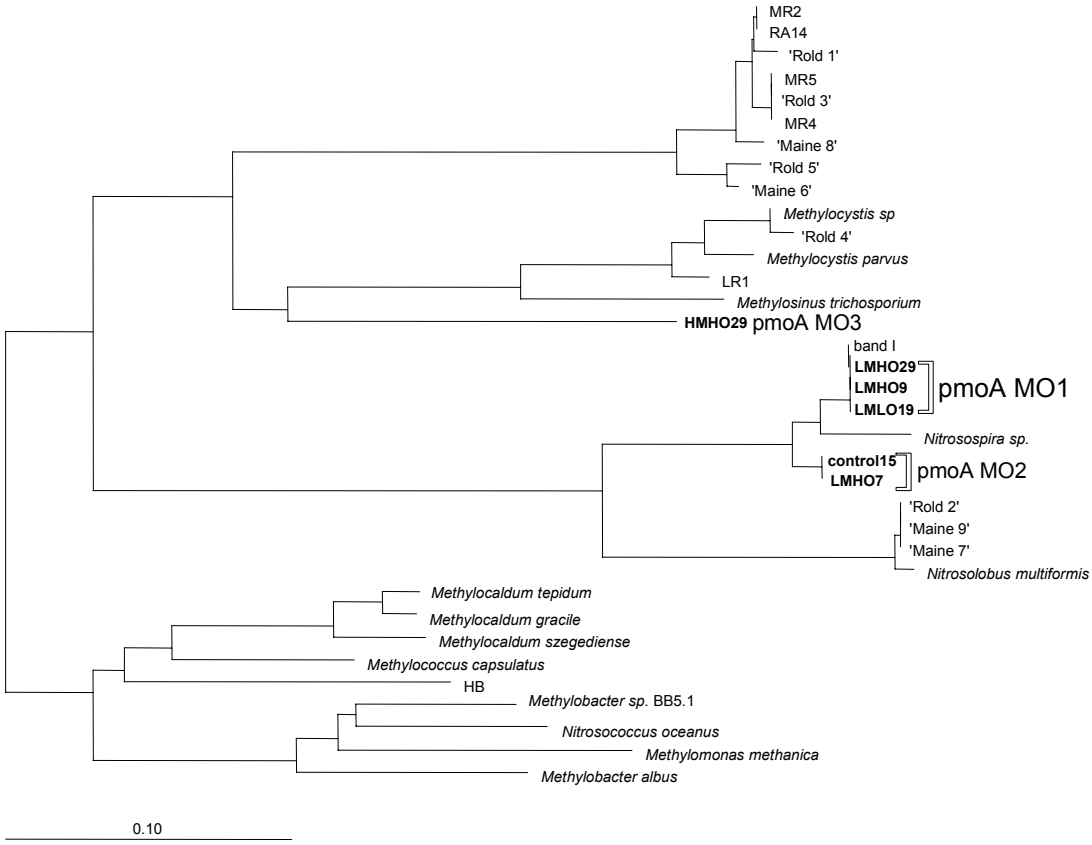

Figure 13. Phylogenetic tree based on the derived amino acid sequences of *pmo*A sequences retrieved from pmoA DGGE bands (see Fig. 10), showing the relationship of the marked DGGE bands to other methanotrophs. The scale bar represents estimated number of changes per amino acid sequence position. Individual DGGE band sequences are labeled according to incubation set-up (HMHO, LMHO, LMLO) and day the sample was taken. mxaf MO 1-3, position of marked DGGE bands; ”MR”, novel *pmo*A DGGE band sequences detected in forest soil; ”Rold”, ”Maine” pmoA clone sequences retrieved from forest soils; band I, amoA DGGE band sequence retrieved from rice field soil; LR1, high affinity type II methanotroph; HB, thermophilic methanotroph.

Discussion

The structure and activity of the indigenous methanotrophic community in rice field soil was effected and changed by incubation at different CH_4 and O_2 mixing ratios. CH_4 oxidation in soils at mixing ratios higher than atmospheric CH_4 is characterized by a two phase process (LeMer et al., 1996; Bender & Conrad, 1995). During the first phase, CH_4 is oxidized at a low initial rate which, after an induction, is followed by a second phase with a several-fold higher rate. The induction can be due to enzyme synthesis, activation of dormant methanotrophs, and/or increase of the methanotrophic population size.

Induction occurred after 5-6 days in incubations HMHO, HMLO and LMLO and after 19 days in LMHO.The increase of 16:1ω8c and 18:1ω8c PLFAs showed that induction coincided with growth and a population increase of type I and II methanotrophs. The methanotrophic biomass increased 9-39-fold when incubated under high CH_4, and 4-5-fold when incubated under low CH_4 mixing ratios, while the oxidation rates increased 20-37-fold

and 7-8-fold during induction, respectively. At CH_4 mixing ratios above 7,000 ppmv CH_4 a population increase was also detected by MPN counts (Bender & Conrad, 1995).

At low CH_4, induction of CH_4 oxidation was repressed by high O_2 for up to 19 days. A complete lack of CH_4 oxidation was reported in rice field soil during a 10-day period without prior preincubation at 10% CH_4 (Bender & Conrad, 1992). Inhibition of CH_4-oxidation by O_2 has been reported before (see Introduction). Rudd et al. (1976; 1980) showed that input of nitrogen (N) relieves the inhibiting effect of O_2 in freshwater lakes leading to the conclusion that it is not the CH_4 oxidation process itself, but the nitrogen fixation process needed under N-limiting conditions that was sensitive to high O_2 (Rudd et al., 1976; Rudd & Taylor, 1980). Only type II & X methanotrophs possess nitrogenase genes and fix N_2 (Hanson & Hanson, 1996). Competition experiments in a continuos-flow bioreactor demonstrated that a type II methanotroph (*M. trichosporium*) dominated under N- and Cu-limiting conditions over a type I methanotroph (*M. albus*) (Graham et al., 1993). Type II methanotrophs might thus be favored in N-limited environments over type I methanotrophs that lack nitrogenase activity (Hanson & Hanson, 1996; Mancinelli, 1995). However, it has recently been shown that N-limitation impedes CH_4 oxidation in rice field soil (Bodelier et al., 2000). Therefore, we used AMS-Medium in our soil incubations to prevent N-limitation. Under these conditions, we found that type I methanotrophs, lacking nitrogenase, were also present and active, indicating that N-limitation was an unlikely reason for the observed repression of induction of CH_4 oxidation in LMHO incubations.

Analysis of the soil by PCR-DGGE revealed changes in methanotrophic population structure at a high level of phylogenetic resolution. PCR-DGGE detects the most dominant members of the target populations (Heuer & Smalla, 1997). In wetland and rice field soil, that are flooded and anoxic most of the time, aerobic methanotrophs are not expected to dominate and have not been detected with the universal primer set targeting all life before (Henckel et al, 1999). Indeed, dominant members of the bacterial community grouped within the CFB-phylum and the gram positive bacteria with high G+C content. However, in soil incubated under high CH_4 (HMHO, HMLO) population "UNIMO2", phylogenetically placed within the type II methanotrophs, became also very pronounced . This population was probably involved in CH_4 oxidation of high CH_4 mixing ratios, since it was not detected in soil incubated under low CH_4 (LMHO, LMLO, ambient). Similarly, type II methanotrophic populations detected an the basis of *pmo*A sequences, e.g.PMOAMO3, were the most dominant methanotrophs under high CH_4 (HMHO). Except for these populations that dominated under high CH_4, the type II methanotrophic community structure was very stable and did not change with time, as shown by DGGE analysis with the specific primer sets MB9α and mxaF. A similarly stable type II methanotrophic community structure had been found

earlier in Italian rice field soil and in the rice rhizosphere, (Henckel et al., 1999; Bodelier et al., 2000).

In contrast to the type II methanotrophic community structure, the type I methanotrophs showed pronounced population shifts depending on the CH_4 and O_2 mixing ratios. These shifts were detected by the MB10γ primer set. Thus, the diversity of type I populations was less in soil incubated under high than under low CH_4. Populations MB10γMO4 and MB10γMO 5 dominated the community under high CH_4 right from the beginning of the incubation, but were not pronounced before day 15 at low CH_4 and were completely absent under ambient air. This result indicates that the growth of these type I methanotrophs was stimulated by high CH_4 mixing ratios.

Not only CH_4, but also O_2 influenced the composition of the type I methanotrophic community structure as depicted with the functional primer mxaF. Type I populations mxafMO1 and mxafMO2 appeared only at low O_2 and high CH_4 (HMLO), but were completely absent at high O_2 and high CH_4 (HMHO). This result indicates that these type I populations thrived under low O_2 and high CH_4, and is in contrast to observations with agar diffusion columns that type I methanotrophs favor high O_2 and low CH_4 mixing ratios (Amaral & Knowles, 1995; Amaral et al., 1995).

Although the PCR-DGGE analyses revealed that the sometimes complex changes among individual populations of both type I and II methanotrophs were caused by both the availability of CH_4 or O_2, the incorporation of $^{14}CH_4$ into specific PLFA fractions showed that the synthesis of either type I or type II methanotrophic biomass was mainly caused by the availability of CH_4 only. Oxygen, in the mixing ratios applied, did not much influence the contribution of type I and II methanotrophs to PLFA synthesis. Type I methanotrophs dominated activity with up to 93% during initial CH_4 oxidation and during the induction phase in all incubations, irrespectively of the CH_4 or O_2 mixing ratio. Contribution by type II methanotrophs was less than 30%, and only after 19 days of incubation and only in soil incubated under high CH_4, mixing ratios increased the contribution by type II methanotrophs to about 50% of total ^{14}C-PLFA synthesis from $^{14}CH_4$. It was previously observed that NH_4^+ additions to the rice rhizosphere stimulated type I methanotrophs relatively more than type II methanotrophs (Bodelier et al., 2000). We moistened the soil with NH_4^+-containing AMS-Medium which might have enhanced the growth of type I methanotrophs.

In various environments, type II methanotrophs have been isolated and enriched more frequently than type I methanotrophs. This result has led to the presumption that type II methanotrophs are more abundant than type I methanotrophs (Hanson & Hanson, 1996). Similarly, most -probable-number (MPN) counts in rice field soil have shown that type II methanotrophs were present in higher cell numbers than type I methanotrophs (Bodelier et al., 2000). PCR-DGGE analyses showed that type II methanotrophs were present even in

anoxic rice field soil that did not oxidize CH_4 (Henckel et al., 1999). Appearance of DGGE bands in a time or spatial series of comparable samples indicated a substantial proliferation of a certain target sequence and thus indicates a drastic population increase (Ferris and Ward, 1997; Henckel et al., 1999). The changes in community structure shown by the PCR-DGGE with five different primer sets indicated that type I methanotrophs probably reacted faster to changing conditions than type II methanotrophs. It was suggested that type I methanotrophs dominate in environments that allow rapid growth of methane-oxidizing-bacteria, while type II methanotrophs that tend to survive better are more abundant in environments where growth rates are periodically restricted (Vecherskaya et al., 1993; Hanson & Hanson, 1996). This and the recent studies in rice field soil support this hypothesis (Henckel et al., 1999; Bodelier et al., 2000). Type II methanotrophs were always present, but proliferated and oxidized under high CH_4 mixing ratios mainly, whereas type I methanotrophs were active and proliferated quickly regardless of CH_4 or O_2 mixing ratios.

Collectively, type II methanotrophs are probably mainly active in the homogeneous conditions of the flooded rice field, at oxic-anoxic interfaces of rice roots and surface layers, where they consume high CH_4 concentrations produced in the anoxic surroundings, whereas type I methanotrophs, with the ability to fast response and quick proliferation under changing conditions, are probably more important for CH_4 destruction when CH_4 production decreases and soil conditions become more heterogeneous, as is the case in drained fields and non-irrigated rainfed rice fields.

Acknowledgments

We thank Paul Bodelier for helpful input to this work, Peter Frenzel for providing the combined FID-TCD-GC, and Hans Scholten for help with the $^{14}CH_4$ production. This work was supported by grant BIO-4-CT-960419 from the European Union, P.R. was further supported by the Danish Technical Research Council.

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3. Die Verteilung der methanotrophen Lebensgemeinschaft im Reisfeldboden nach Drainage

In dieser Arbeit wurde gezeigt wie sich die methanotrophe Lebensgemeinschaft nach Drainage eines gefluteten Reisfeldbodens verändert. Typ II MOB scheinen im Reisfeldboden unter anoxischen Bedingungen gut überdauerungsfähig zu sein, denn bereits in den noch wassergesättigten und anoxischen Bodenschichten waren kurz nach der Drainage Typ II MOB vorhanden. Die Populationsstruktur der Typ II MOB veränderte sich nur geringfügig nach der Drainage.

Typ I MOB hingegen waren in den noch wassergesättigten Bodenschichten einen Tag nach der Drainage kaum oder nicht nachweisbar. Während der Drainage kam es zu einer starker Stimulation und Wachstum von Typ I MOB. Im drainierten Boden waren in allen untersuchten Bodenschichten Typ I MOB dominant vorhanden. Die Diversität der Populationsstruktur von Typ I MOB veränderte sich in fast jedem der getesteten 2-mm Bodensegmente und zeigte, dass die Populationen der Typ I MOB wahrscheinlich an spezielle Umweltbedingungen angepasst sind.

Submitted to FEMS Microbiology Ecology

Vertical distribution of the methanotrophic community after drainage of rice field soil

Thilo Henckel, Udo Jäckel, Ralf Conrad*

Abstract

The budget of atmospheric CH_4 is of concern, since CH_4 is one of the important greenhouse gases that affect Earth's climate. Anoxic soils like flooded rice fields are major sources while oxic upland soils are a major sinks of atmospheric CH_4. Nevertheless, CH_4 is also consumed in rice fields where up to 90% of the produced CH_4 is oxidized in a narrow oxic zone around the rice roots and at the soil-surface layer before it escapes to the atmosphere. After 1 day drainage of rice field soil, CH_4 oxidation was detected in the top 2 mm soil layers, but after 8 days drainage the zone of CH_4 oxidation extended to 8 mm depth. Simultaneously, the potential for CH_4 production decreased with time of drainage, but some CH_4 production was still detectable after 8 days drainage throughout the soil profile. The vertical distribution of the methanotrophic community was also monitored after 1 and 8 days drainage using PCR-DGGE with different PCR primer sets targeting two regions that are relatively specific for methylotrophic α- and γ-*Proteobacteria* on the 16S rRNA gene, and targeting two functional genes coding for subunits of key enzymes in all methanotrophs, i.e. the genes for the particulate methane monooxygenase (*pmo*A) and the methanol dehydrogenase (*mxa*F). Drainage stimulated the methanotrophic community. Eight days after drainage new methanotrophic populations appeared and a distinct methanotrophic community developed. The population structure of type I and II methanotrophs was differently affected by drainage. Type II methanotrophs were present throughout the soil core directly after drainage (1 d), and the community composition remained largely unchanged with depth. Only two new type II populations appeared after 8 days of drainage. Drainage had a more pronounced impact on the type I methanotrophic community. Type I populations were not or only weakly detected one day after drainage. However, after 8 days of drainage a wide diversity of type I populations was detected which, however, were not evenly distributed throughout the soil core but dominated at different depths. A distinct type I community structure had developed within each soil section between 0 and 20 mm soil depth, indicating the widening of suitable habitats for methanotrophs in the rice field soil within one week after drainage.

Introduction

Flooded rice fields are a major source of the greenhouse gas CH_4, thus contributing to global warming. The atmospheric CH_4 concentration (1.8 ppmv) has more then doubled during the last 200 years (ref). Although more than 90% of the CH_4 production is of biogenic origin, produced by anaerobic methanogenic archaea, the source strenght is directly influenced by anthropogenic activity, such as by growing wetland rice (Conrad, 1997). About 80% of the rice harvest is grown under the more productive flooded conditions (wetland rice) which produce about 25 to 60 Tg CH_4 yr^{-1} (Conrad, 1997; Neue, 1997). The estimated increase in demand for rice by the year 2020 of 65% calls for the development of strategies to reduce and control CH_4 emissions from wetland rice (Neue, 1997). Hence, basic knowledge of the underlying processes is essential.

One strategy to mitigate CH_4 emission is a short intermittent drainage of the flooded fields (Sass et al., 1992; Yagi et al. 1996). Methanogenesis not only stopps immediately after anoxic reduced soil is drained or aerated, but in addition is suppressed after re-flooding for a prolonged period, since the aeration of the soil during drainage results in the re-oxidation of electron acceptors such as Fe^{3+} and SO_4^{2-} which then inhibit methanogenesis (Ratering & Conrad, 1998; Sigren et al. 1997; Achtnich et al., 1995).

In flooded rice fields methane oxidation is also an important regulator of CH_4 emissions. Of the produced CH_4 about 30-90% is oxidized in the upper 2-mm oxic surface layer or in the oxic rhizosphere and thus is not emitted into the atmosphere (Conrad & Rothfuss, 1991, Frenzel et al., 1992; King, GM, 1996; van der Gon et al., 1996; Bosse & Frenzel, 1997, 1998). Oxygen is a prerequisite for CH_4 oxidation. In non-saturated or aerated rice field soil CH_4 mixing ratios higher than atmospheric are readily consumed by methanotrophs (Henckel et al., 1998). Both type I and II methanotrophs are present and active in rice field soils (Henckel et al., 1999, in prep). In wetland soils, CH_4, O_2 and NH_4^+ are proximal factors controlling CH_4 oxidation (Schimel et al., 1993). In rice field soil, especially CH_4 and NH_4^+ availability were shown as the key factors influencing methanotrophic community structure and activity (Bodelier et al., 2000; Henckel et al., in prep). Under certain conditions, even low atmospheric CH_4 concentrations can be oxidized by samples of rice field soil (Bender and Conrad 1992; Thurlow et al., 1995; Jäckel et al., 2000).

In flooded rice field soil, methanotrophic activity is confined to a very narrow zone at the oxic/anoxic interfaces, serving as a filter for CH_4. Drainage alters soil conditions dramatically and may effect the distribution and composition of the methanotrophic community, thus influencing CH_4 uptake. We therefore characterized the vertical distribution of the methanotrophic population structure during drainage of rice field soil cores.

Materials and Methods

Incubation of the rice field soil cores. Air dried and sieved (2 mm) rice field soil from Vercelli (Italy) was filled in polyethylene-boxes. The boxes (unplanted microcosms) were submersed in water and incubated in a greenhouse under day-night alternation for 6 weeks.

The microcosms were taken out of the water and drained through 6-mm holes in the bottom of the microcosm. One and eight days after drainage five soil cores were cut with a corer (∅6 cm) from the microcosms. One core was collected to determine potential methane production, three cores were used to measure methane oxidation activity, and a fifth core (∅1.6 cm) was immediately frozen at -20 °C and sectioned for molecular analysis of the microbial community.

Methane oxidation /potential methane production. Rice field soil cores were sectioned into 2-mm soil layers. Soil sections were transferred into 150-ml glass flasks closed with latex stoppers. The oxidation of ambient CH_4 mixing ratios were measured by repeatedly taking gas samples. Methane was analyzed by gas chromatography using a flame ionization detector. Methane oxidation rate constants were determined by exponential curve fitting (Origin 5.0, Microcal), and CH_4 oxidation rates were calculated by multiplying the rate constants with a CH_4 concentration of 1.75 ppmv (ambient CH_4 mixing ratio). Potential CH_4 production was determined analogously, by incubating a soil sample under N_2 and measuring the increase of CH_4 with time. Production rates of CH_4 were calculated by linear regression analysis.

DNA extraction. A rice soil core (∅1.6 cm) was sectioned into 300 µm layers with a mycrotom at -20°C. the soil sections were combined in 2-mm steps and mixed.DNA extraction from rice field soil and from pure cultures of methanotrophs was modified after Moré *et al*. (1994) and thoroughly described in Henckel et al. (1999).

PCR amplification. For PCR amplification we used three SSU rRNA-based primer sets, i.e. an ”universal” primer set targeting all life and the primer sets "MB10γ" and "MB9α", targeting methylotrophic γ- and α-*Proteobacteria*, respectively. Further, two functional primer sets were used, i.e. "pmoA" and "mxaF", targeting the particulate methane monooxygenase (pMMO) and the methanol dehydrogenase (MDH) key-enzymes present in all methanotrophs, respectively (Henckel et al., 1999). All used primer pairs had a GC-clamp attached to the 5´end of the forward or reverse primer for subsequent DGGE analysis (Henckel et al., 1999).

The primers and the PCR conditions for each primer pair were recently described in detail in Henckel et al. (1999). The thermal cycling profile consisted of an initial denaturation of 3 min at 94ºC, followed by 30 to 32 cycles and 5 min final elongation at 72ºC (Table 1). For PCR amplification with the primer sets pmoA and Universal, PCR conditions were optimized using a touchdown program (Table 1).

Aliquots (5 µl) of PCR products were analyzed by electrophoresis on 3% agarose gels, stained with ethidium bromide.

Table 2. PCR and DGGE conditions for the various primers used. GC clamps used are indicated by stars *cgcccgccgcgccccgcgcccggcccgccgcccccgcccc, and **ccccccccccccgccccccgccccccgccccccgccgccc

Primer	Target Gene	PCR			DGGE
		temperature profiles	cycles	Reaction mix	gradient time voltage
UNI 533f/907r*	16S rRNA	94°C, 30 s 60-50°C, 30 s 72°C, 45 s	30	1,5 mM MgCl	35-70 % 5 h 150V
MB9α 9α f / 533r*	16S rRNA	94°C, 3 min 94°C, 1 min 60°C, 30 s	31	Premix F	20-70 % 5 h 150 V
MB10γ 10γ f / 533r*	16S rRNA	94°C, 1 min 60°C, 30 s 72°C, 1 min	31	Premix F	40-70 % 5 h 150 V
pmoA A189/A682**	α-subunit of the pMMO *pmo*A	94°C, 1 min 62-55°C, 30 s 72°C, 45s	30	Premix F	35-80 % 6 h 200V
mxaF 1001/ r1557**	α-subunit of the MDH *mxa*F	94°C, 1 min 55°C, 30 s 72°C, 1 min	31	Premix E	35-70 % 5 h 150V

PCR-DGGE. PCR products were separated using a DCode System (Bio-Rad, Munich, Germany) on 1-mm thick polyacrylamide gels (6.5% w/v acrylamide:bis acrylamide (37.5:1); Bio-Rad) prepared with and electrophoresed in 0.5 × TAE, pH 7.4 (0.04 M Tris-base, 0.02 M sodium-acetate, 1 mM EDTA) at 60°C, and constant voltage (Henckel et al., 1999). A denaturing gradient of 80% (vol/vol) denaturant corresponded to 6.5% acrylamide, 5.6 M urea and 32% deionized formamide. Gels were poured on GelBond PAG film (FMC Bioproducts, Rockland, ME, USA) to avoid gel distortion. Running conditions are summarized in Table 1. Gels were stained with 1:50,000 (vol/vol) SYBR-Green I (Biozym, Hessisch-Oldendorf, Germany) for 30 min, and scanned with a Storm 860 phosphor imager (Molecular Dynamics, Sunnyvale, CA, USA). The scanned DGGE gels were, as a whole, digitally enhanced with ImageQuant 5.0 (Molecular Dynamics, Sunnyvale, CA, USA) or Adobe-PhotoShop 5.0 (Adobe Systems Incorporated).

For further analysis, DGGE bands were visualized in the SYBR Green I-stained gels with blue light (λ > 400 nm) using a Dark Reader transilluminator (Clare Chemical Research, Ross on Wye, UK). Individual DGGE bands were then excised, reamplified, and reanalyzed by DGGE to verify band purity (Henckel et al., 1999).

Cloning of MB9α DGGE bands. PCR products of excised and reamplified MB9α bands exhibited multiple DGGE bands when reanalyzed in DGGE. Therefore these PCR products were cloned using the pGEM-TEasy cloning kit (Promega, Madsion, WI). Randomly selected clones (8 per DGGE band) were amplified with a primer set targeting the vector sequence (Rotthauwe *et al*., 1997). The purified PCR-products of the cloned fragments containing the vector sequences were again amplified with the MB9α primer set to regain the original MB9α 16S rDNA fragment.

These MB9α PCR products were reanalyzed on DGGE together with the original MB9α PCR product amplified from rice soil template. All cloned MB9α PCR products showed single bands on DGGE. Bands corresponding with a respective rice soil band were sequenced.

Sequencing/phylogenies. Reamplified PCR products of excised DGGE bands were purified using the EasyPure DNA purification kit (Biozym, Hessisch-Oldendorf, Germany). Mixing ratio and purity of PCR products were determined by absorption at 260 nm and 280 nm of a 1:20 dilution in H_2O with a GeneQuant spectrophotometer (Pharmacia Biotech, Upsala, Sweden). Sequencing reactions were performed using the ABI Dye-terminator cycle sequencing kit (Perkin Elmer Applied Biosystems) as specified by the manufacturer. Cycle sequencing products were purified from excess dye terminators and primers using Microspin G-50 columns (Pharmacia Biotech, Freiburg, Germany), and analyzed with an ABI 373 DNA sequencer (Perkin Elmer Applied Biosystems).

Sequences were analyzed and edited using the Lasergene software package (DNASTAR, Madison, WI, USA). The inferred amino acid sequences of the gene fragments of *pmo*A and *mxa*F were manually aligned with sequences retrieved from the GenBank database. SSU rDNA sequences were aligned and phylogenetically placed with the ARB software package (Strunk and Ludwig, 1996). The partial SSU rDNA sequences were added to a validated and optimized tree of about 5000 complete SSU rDNA sequences, while keeping the overall topology constant using the maximum parsimony method (Ludwig et al., 1998). Phylogenetic trees were constructed by using distance matrix and maximum parsimony methods supplied by the ARB software package (http://www.biol.chemie.tu-muenchen.de/pub/ARB/). On the nucleic acid level, evolutionary distances between pairs

of sequences were calculated by using the Jukes-Cantor, and Felsenstein equations (Jukes and Cantor, 1969; Felsenstein, 1993) implemented in the ARB package.

Accession numbers. Sequences of partial *pmoA* and *mxaF* gene fragments, and of 16S rRNA gene fragments of excised DGGE bands have been deposited in GenBank under accession numbers xxxxx, and xxxxxx

Results

CH_4 oxidation and production. One day after drainage the water content of the soil was still 121% WHC in the top 2-mm surface layer decreasing to 66% WHC at 20 mm depth (Fig. 1A). Ambient CH_4 was oxidized only in the top 2-mm soil layer (Fig. 1A). However, CH_4 production occurred throughout the soil core when incubated under anoxic conditions. After 8 days drainage, the water content had dropped to 52% WHC at the surface and 40% WHC at 20 mm soil depth, and atmospheric CH_4 was consumed between 0 and 8 mm depth (Fig. 1B). The potential for CH_4 production under anoxic conditions had decreased dramatically, but still was detectable throughout the soil core (Fig. 1B).

PCR amplification. DNA was extracted from 2-mm sections collected from the drained soil cores one and eight days after drainage, and used in PCR amplification. PCR products of the expected sizes were obtained by amplification with the functional primer sets pmoA and mxaF, and the 16S rDNA primer sets MB9α, MB10γ and Uni. The primer sets (Uni, MB9α, pmoA) targeting the 16S rDNA of all life and type II methanotrophs and the pMMO produced PCR products with similar yields from all samples, indicating that the template DNA was readily amplifiable and not subjected to biases caused by humic acids and other PCR-inhibiting substances. On the other hand, amplifications with the primer sets (mxaF, MB10γ) targeting the 16S rDNA of type I methanotrophs and the methanol dehydrogenase resulted in high PCR product yields only when DNA retrieved after 8 days of drainage was used as template, whereas only low (MB10γ) or no PCR product (mxaF) was obtained when DNA was extracted one day after drainage and from soil below 2 mm soil depth (results not shown).

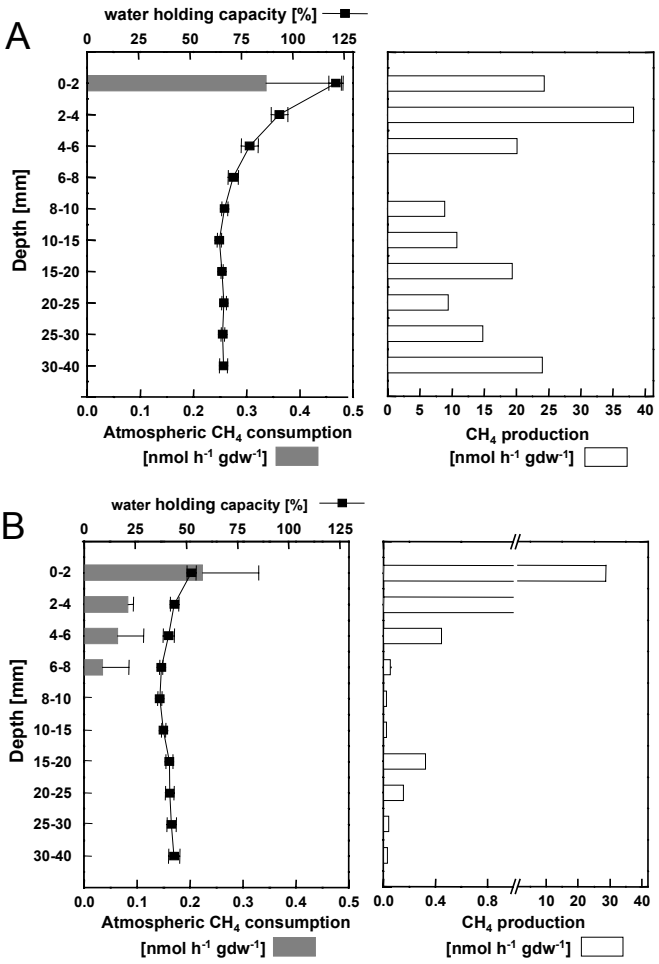

Figure 1. Vertical profiles of atmospheric CH_4 consumption, water-holding capacity and CH_4 production in rice field soil cores one (A) and 8 days (B) after drainage; means ±SD of n=3.

MB9α rDNA community pattern. The DGGE analysis of PCR products amplified with the MB9α primer set showed a qualitatively similar banding pattern irrespectively of the soil depth and the time after drainage (Fig. 2). DGGE band MB9α-RC5 was the most prominent band and appeared in all samples with similar intensity. The same was the case with bands RC6 and RC7 which, however, were not as intensive as RC5. DGGE bands RC1, RC2, and RC3 were more distinct after 8 days than after 1 day of drainage. They all were found in the entire soil core (0-20 mm depth) except band RC3 which was not detected below 8 mm soil depth (Fig. 2).

Sequence analysis of cloned MB9α bands showed that all bands grouped within the α-*Proteobacteria* (Fig.2). DGGE bands RC5 and RC6 grouped within the type II methanotrophs close to *Methylosinus/Methylocystis*. The most dominant Band RC5 was identical to sequences detected before in the rhizosphere of rice and in most-probable-number (MPN) enrichments from the rice rhizosphere (RRII4, RRII3, ERRII2) (Bodelier et al., 2000). Band RC6 was almost identical. Bands RC1, RC2, RC3 and RC7, on the other hand, were related to *Caulobacter* species*, Sphingomonas* species*, Methylobacterium* species and *Rhodopseudomonas acidophila*, respectively (Fig. 2).

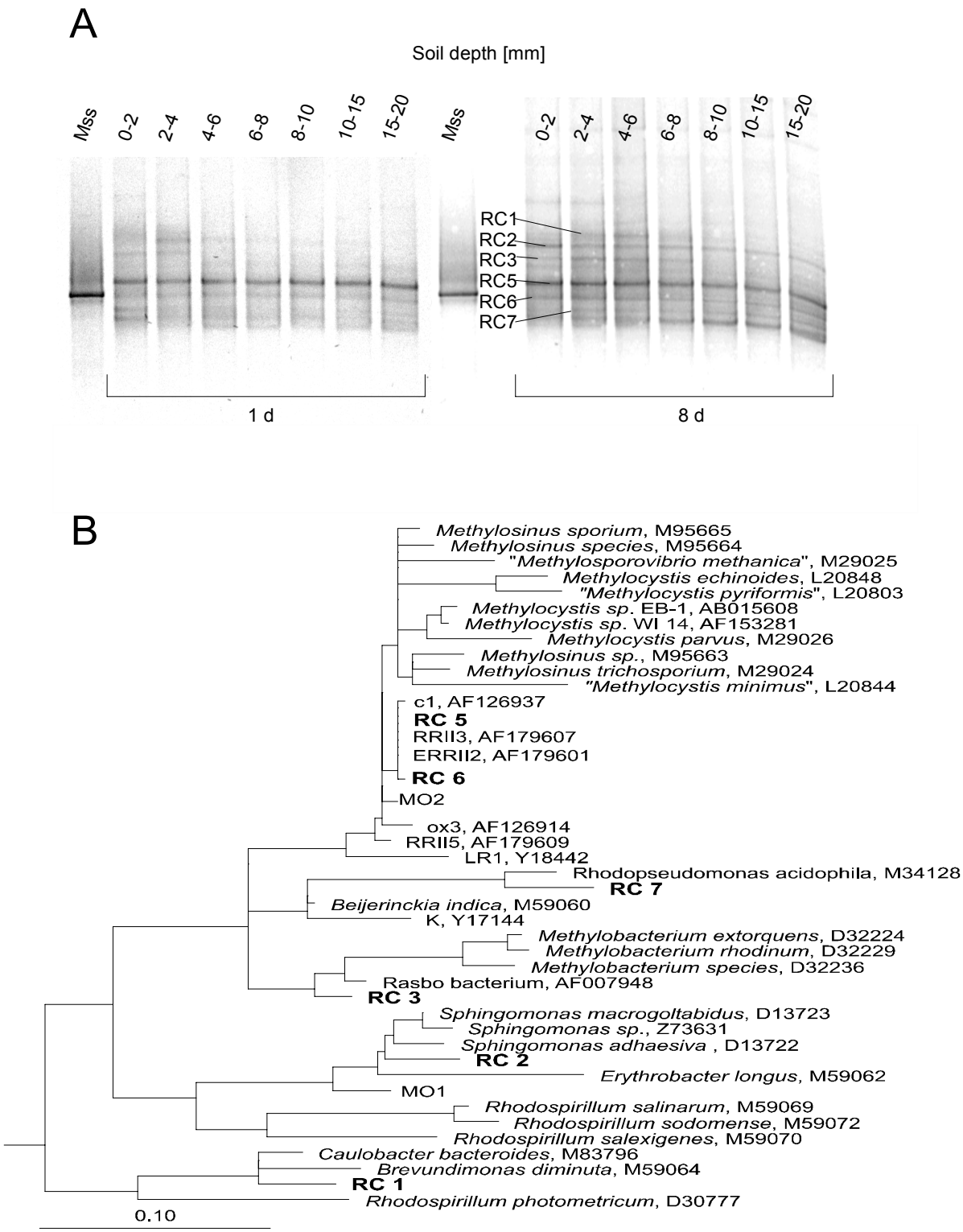

Figure 2. DGGE banding pattern (A) obtained after amplifying DNA extracted from rice field soil cores with the 16S rDNA primer set MB9α targeting type II methylotrophs. Soil samples for DNA extraction were taken one day and 8 days after drainage at indicated soil depths. Bands of marked position were cloned and sequenced: Mss: *Methylosinus sporium*. The phylogenetic tree (B) was constructed with the partial 16S rDNA sequences and related sequences from α-*Proteobacteria*. The partial sequences were added by maximum parsimony to a verified tree without changing the tree topology; RC1, RC3, RC5 and RC6:, clones corresponing to the marked positions MB9α-RC1, etc.; LR1: high affinity type II methanotroph; K: acidophilic methanotroph; c1, ox3: clones retrieved from rice field soil incubated without and with CH_4, respectively; RRII3, ERRII2: clones retrieved from rice rhizosphere soil or from enrichment cultures.

MB10γ rDNA community pattern. The DGGE analysis of PCR products amplified with the MB10γ primer was not clearly resolved one day after drainage and below 4 mm soil depth due to low PCR product yield (Fig. 3). DGGE band MB10γ-RC8 was the most pronounced band, but only in the top 4 mm soil layer. After 8 days of drainage, however, PCR products were obtained at high yields, and the DGGE analysis showed a distinct banding pattern changing with soil depth (Fig. 3). The community structure was different in almost each 2-mm soil section. The highest number of DGGE bands was detected between 4 and 8 mm depth, i.e. the depth to which activity of atmospheric CH_4 oxidation was detected (Fig.1). In the soil layers above 4 mm and below 8 mm depth a smaller number of DGGE bands was detected indicating fewer microbial populations. Bands RC1 to RC5 were missing or only barely detectable in the top layer between 0 and 2 mm depth, but intensified below 4 mm and were most pronounced below 10 mm depth (Fig. 3). In contrast, band RC8 was most pronounced and intense at the surface and slowly disappeared in deeper soil layers, being absent below 10 mm depth, while band RC7 was most intensive between 6 and 15 mm depth (Fig. 3).

Sequence analysis revealed that all bands tested grouped within the γ-*Proteobacteria* (Fig. 3). Bands RC1 and RC3 (most pronounced below 2 mm depth) but also bands RC5 and RC6 (below 6 mm depth) grouped among the type I methanotrophs, closest to previously described clones (RRI) from the rice rhizosphere (Bodelier et al., 2000) and to *Methylobacter* species (Fig. 3). Bands RC7 and RC8, on the other hand, were rather related to *Thiocapsa roseopersicina* or *Methylophaga* species as the closest relatives (Fig. 3).

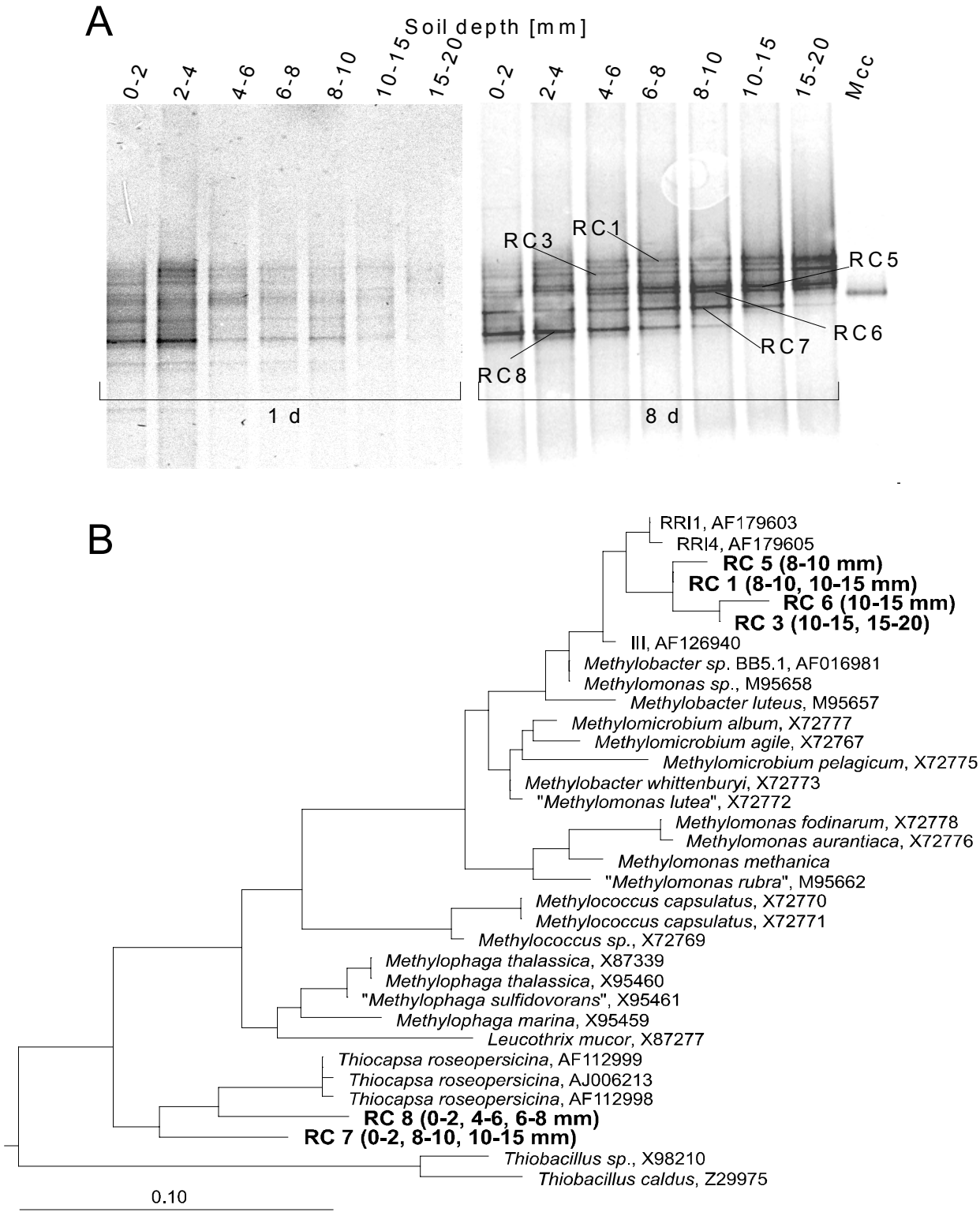

Figure 3. DGGE banding pattern (A) obtained after amplifying DNA extracted from rice field soil cores with the 16S rDNA primer set MB10γ targeting type I methylotrophs. Soil samples for DNA extraction were taken one and 8 days after drainage at indicated soil depths. Bands of marked position were sequenced; Mcc: *Methylococcus capsulatus*. The phylogenetic tree (B) was constructed with the partial 16S rDNA sequences and related sequences from γ-*Proteobacteria*. The partial sequences were added by maximum parsimony to a verified tree without changing the tree topology;. RC1, RC3, RC5, RC6, RC7 and RC8: marked DGGE bands MB10γ-RC1, etc., (mm): soil depth from which the band was retrieved; RRI1 and RRI4: clones retrieved from rice rhizosphere soil; III: MB10γ DGGE band sequence retrieved from rice field soil incubated with CH_4.

mxaF DGGE community pattern. The DGGE analysis of PCR products amplified with the functional primer set mxaF showed after 8 days of drainage one dominant band mxaF-RC2 which was present in all depth layers (Fig. 4). In the surface layers between 0 and 4 mm depth, a second faint band RC1 was visible. Sequence analysis of the *mxa*F bands revealed that both RC1 and RC2 grouped among the type II methanotrophs, closest to the genus *Methylocystis* (Fig. 4). Band RC 2 was identical to the *mxa*F sequence „band II“ retrieved from rice field soil incubated under CH_4 (Henckel et al., 1999). Band RC1 was identical to *mxa*F sequence „con11“ retrieved from the rice rhizosphere (Henckel & Bodelier, unpublished data).

pmoA DGGE community pattern. The DGGE analysis of PCR products amplified with the functional primer set pmoA showed changes of the banding pattern with depth and time of drainage. One day after drainage, bands pmoA-RC1, RC2 and RC4 were detected in surface layers between 0 and 4 mm soil depth (Fig. 5). Below 4 mm soil depth, bands RC2 and RC4 were faint and were absent below 15 mm soil depth. After 8 days of drainage, band intensity of RC4 had increased and this band was most pronounced down to 10 mm soil depth. Between 2 and 4 mm depth an additional band appeared, which was not detected in any other soil sample (Fig. 5). Band RC1 was the most dominant band between 10-15 mm depth. Below 15 mm soil depth, no DGGE bands were detected.

Sequence analysis revealed that all bands tested (RC1, RC2,RC4) grouped among the type I methanotrophs, closest to the genus *Methylococcus* (Fig. 59. Band RC1 was similar to pmoA population „band II“ detected in rice field soil incubated under CH_4 (Henckel et al., 1999).

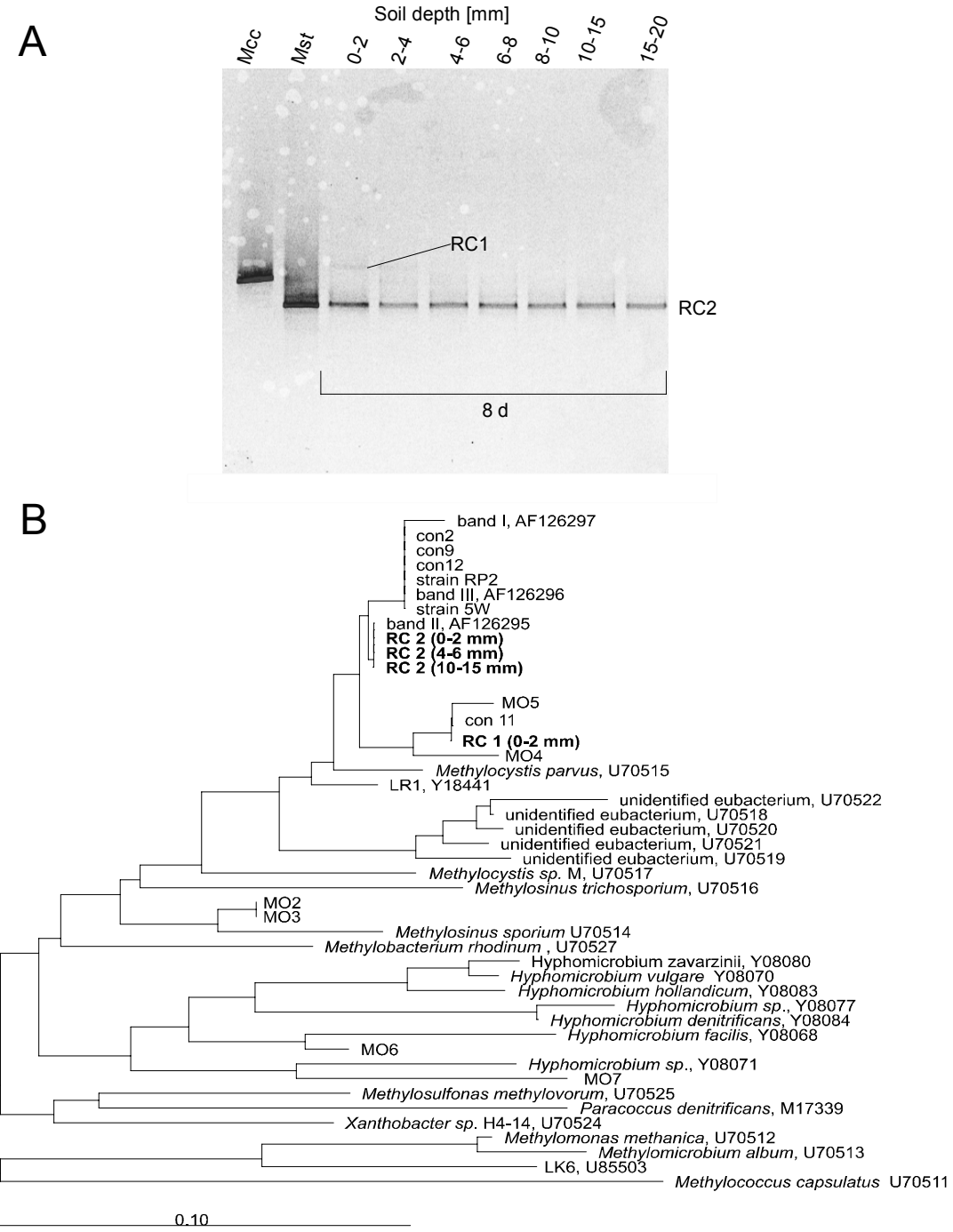

Figure 4. DGGE banding pattern (A) obtained after amplifying DNA extracted from rice field soil cores with the functional primer set mxaF targeting the gene of the α-subunit of the MDH present in all methylotrophs. Soil samples for DNA extraction were taken 8 days after drainage at indicated soil depths. Bands of marked positions were sequenced; Mcc: *Methylococcus capsulatus*; Mst: *Methylosinus trichosporium*. The phylogenetic tree (B) was based on the derived amino acid sequences of *mxa*F sequences retrieved from mxaF DGGE bands showing the relationship of the marked DGGE bands to other methylotrophs. The scale bar represents estimated number of changes per amino acid sequence position; RC1, RC2: marked DGGE bands mxaF-RC1 and mxaF-RC2, (mm): soil depth from which the band was retrieved; con2, con9, con11, con12:, mxaf DGGE band sequences retrieved from rice rhizosphere soil (Henckel & Bodelier, unpublished data); band I, band II, band III: mxaf DGGE band sequences retrieved from rice field soil incubated with CH_4; MO2, MO3, MO4, MO5, MO6, MO7: mxaF DGGE band sequences retrieved from rice field soil incubated under different O_2 and CH_4 mixing ratios (Henckel et al., in prep); LR1: high affinity type II methanotroph; LK6: unclassified soil isolate; strain RP2, 5W: type II methanotrophs enriched from the rice rhizosphere (Frenzel et al., unpublished data).

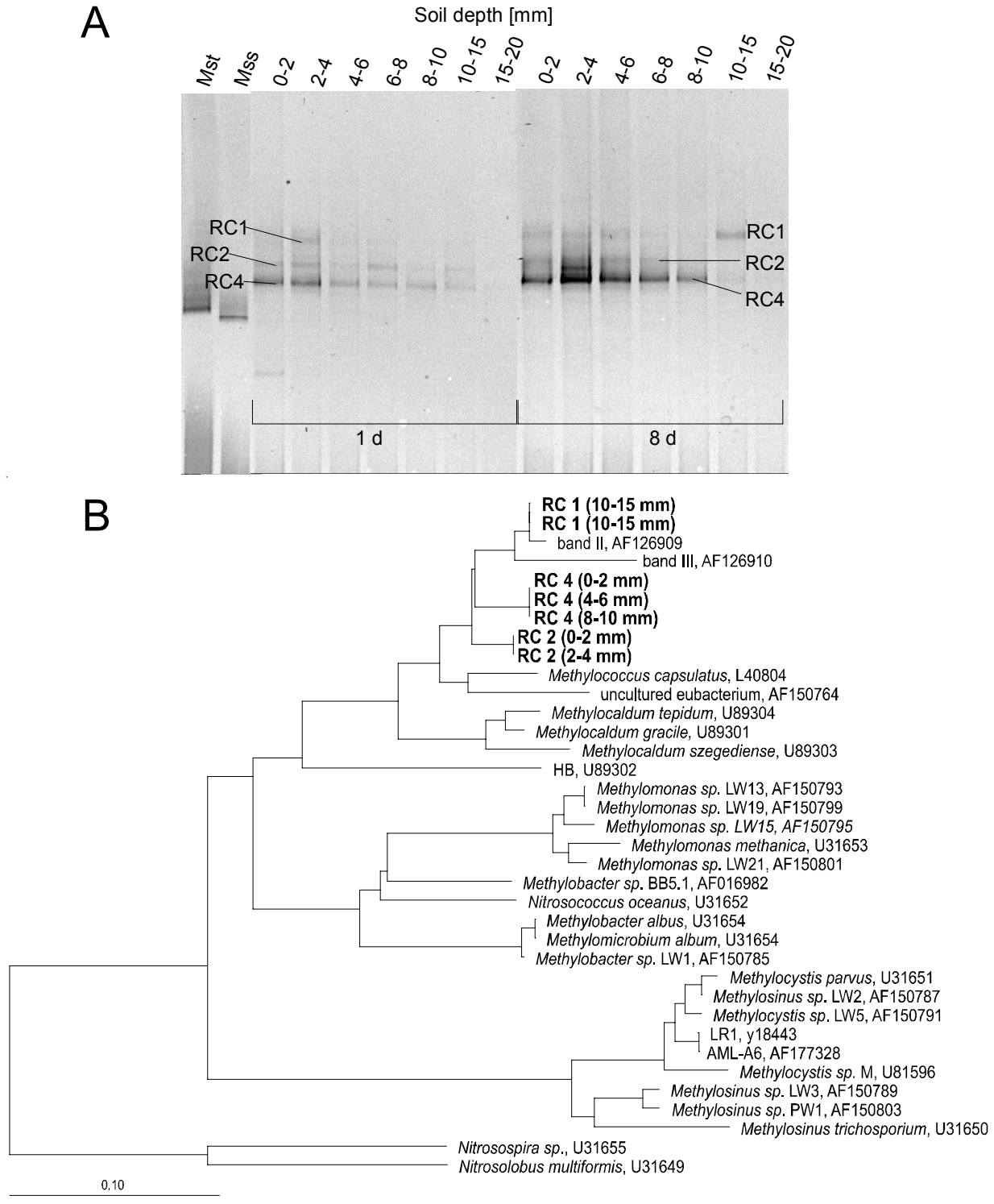

Figure 5. DGGE banding pattern (A) obtained after amplifying DNA extracted from rice field soil cores with the functional primer set pmoA targeting the gene of the α-subunit of the pMMO present in all methanotrophs. Soil samples for DNA extraction were taken one and 8 days after drainage at indicated soil depths. Bands of marked position were sequenced; Mst: *Methylosinus trichosporium*; Mss: *Methylosinus sporium*. The phylogenetic tree (B) was based on the derived amino acid sequences of *pmo*A sequences retrieved from pmoA DGGE bands, showing the relationship of the marked DGGE bands to other methanotrophs and ammonium-oxidizers. The scale bar represents estimated number of changes per amino acid sequence position; RC1, RC2, RC4: marked DGGE bands pmoA-RC1, etc., (mm): soil depth from which the band was retrieved; band II, band III: pmoA DGGE band sequence retrieved from rice field soil; LR1: high affinity type II methanotroph; AML-A6: type II methanotroph isolated from landfill soil; HB: thermophilic methanotroph.

Discussion

Methane oxidation activity, measured as uptake of atmospheric CH_4, was confined to the top 2 mm layer of flooded rice field soil. Drainage of soil extended the vertical distribution of CH_4 oxidation activity down to 8 mm soil depth. With decreasing soil water content, the methanotrophic community also extended its zonation, presumably as O_2 became available to deeper soil layers.

Type II methanotrophs were detected throughout the soil core directly after drainage as shown with the MB9α primer set. The populations MB9α-RC5 and MB9α-RC 6 were identical and very closely related to populations RRII3, RRII4, ERRII2 and c1 which had previously been detected in the rice rhizosphere of flooded rice microcosms, bulk soil and rice field soil incubated under CH_4 (Henckel, et al., 1999; Bodelier et al., 2000). The presence of the same type II methanotrophs in various studies indicates that these populations are dominant members of the type II methanotrophic community in Italian rice field soil and survive well periods of flooding and anoxic conditions. Methanotrophs have been shown to survive better under anoxic than oxic conditions (Roslev & King, 1994). We conclude that type II methanotrophs represented by MB9α-RC5 and MB9α-RC6 can generally be detected in soil layers even when CH_4 oxidation is not active.

Other populations that were detected with the MB9α primer set, e.g. MB9α-RC1 and MB9α-RC3 seemed to be confined to the upper soil layers. However, these populations were phylogenetically affiliated with non-methanotrophic methylotrophs, such as *Caulobacter* or *Methylobacterium* species and thus, were probably not involved in CH_4 oxidation.

We have repeatedly found that the *mxa*F gene was not or just barely detectable in soil that was not actively oxidizing CH_4 (Henckel et al., 1999; in prep. ; Henckel & Bodelier, unpublished data). Here, we found that just one day drainage the *mxaF* gene was only detectable in the top 2-mm soil layer. After 8 days drainage, however, two mxaF-RC1 and mxaF-RC2, both related to *Methylocystis* species, became detectable. These two populations probably belonged to type II methanotrophs which had been detected before, i.e. band II in Henckel et al. (1999). However, it should be noted that the *mxaF* gene is present in all methylotrophic species irrespectively of whether they are able to oxidize CH_4. Therefore, we cannot be sure that the populations represented by mxaF-RC1 and mxaF-RC2 are able to oxidize CH_4. The populations detected with the pmoA primer set have the gene for the pMMO and thus should be able to oxidize CH_4. Indeed, *pmoA* was mainly detected in the upper soil layers. After 8 days of drainage, pmoA-RC2 and pmoA-RC4 were not detectable below 8-10 mm depth, which corresponds to the soil depth down to which oxidation of atmospheric CH_4 was detectable. Similarly after 1 day of drainage, the pmoA DGGE bands were most pronounced in the top 4 mm soil layers and oxidation of atmospheric CH_4 was detectable down to 2 mm depth. However, it should be noted that CH_4 oxidation activity,

albeit at elevated CH_4 concentrations, may also have occurred in soil layers that were unable to oxidize CH_4 at atmospheric concentrations. This possibility may explain, why faint pmoA DGGE bands were also detectable in soil layers that were unable to oxidize atmospheric CH_4. The sequenced bands pmoA-RC1, pmoA-RC2 and pmoA-RC4 were related to the genus *Methylococcus*, a type I methanotroph (also called type X; Hanson and Hanson 1996) containing both the particulate (pMMO) and the soluble methane-monooxygenase (sMMO). *Methylococcus* contains multiple *pmo*A gene copies, hence the number of *pmo*A-bands does not necessarily correlate to the number of populations (Semrau et al., 1995; Henckel et al., 1999).

Interestingly, in this study only the *pmoA* of type I methanotrophs was detected, although both types of methanotrophs have been detected before with the pmoA primer set (Henckel et al., in press; Henckel et al., 1999; Dunfield et al., 1999). Similarly, only the *mxaF* of type II methanotrophs were detected, although *mxaF* of type I methanotrophs had been detected before in Italian rice field soil (Henckel et al., in press; Henckel et al., 1999; Dunfield et al., 1999). We have no explanation for this discrepancy.

Consistent with the dominance of type I methanotrophic *pmoA* populations, drainage also stimulated the type I methanotrophic community that was represented by the 16S rDNA amplified by the MB10γ primer set. One day after drainage, distinct type I methanotrophs were not detectable below 4 mm soil depth and were only barely detectable in the top-soil layers. However, after 8 days of drainage the type I methanotrophic community structure revealed distinct DGGE patterns with different type I populations dominating in different soil layers. At this time, an opposite gradient of CH_4 and O_2 concentrations should exist in the soil core and the type I populations probably responded by establishing at the respective optimum. Interestingly, the diversity of type I populations was not so pronounced in the top2-mm soil layer, in which CH_4 oxidation activity was highest, but instead diversity was most pronounced in deeper soil. The detected and sequenced MB10γ populations were closest related to *Methylobacter* species. This is in contrast to the pmoA populations which were closest related to *Methylococcus* species. Again, however, some of the MB10γ populations were also found in soil layers below the depth at which atmospheric CH_4 was oxidized.Recently, we have shown that type I methanotrophs were able to proliferate rapidly and dominate CH_4 oxidation in aerated rice field soil when CH_4 was supplied at low mixing ratios, whereas type II methanotrophs were only proliferated at high CH_4 mixing ratios (Henckel et al., in prep). However, type II methanotrophs were the dominant community in flooded rice soil (Bodelier et al, 2000). It has been suggested that type I methanotrophs dominate in environments that allow rapid growth of methane-oxidizing-bacteria, while type II methanotrophs are more abundant in environments where growth rates are periodically restricted (Vecherskaya et al., 1993; Hanson & Hanson, 1996). Our results are

consistent with this hypothesis. The community shifts upon drainage were less pronounced with type II than with type I methanotrophs, indicating that type II methanotrophs had a better ability to survive but did not react rapidly to environmental conditions. Flooded rice field soil is characterized by more relatively homogeneous soil conditions that are mostly adverse to CH_4 oxidation. However, high CH_4 production rates in deep soil supplies high CH_4 concentrations at the soil surface layer where O_2 is also available and thus provides the optimum conditions for the proliferation of type II methanotrophs. After drainage, however, soil conditions become increasingly heterogeneous, CH_4 mixing ratios decrease and thus, type I methanotrophs are favoured.

In summary, our results show that drainage of rice fields affects the methanotrophic community structure, increasingly favoring the development of type I methanotrophs.

Acknowledgements

We thank Bianca Wagner for excellent assistance with cloning and sequencing. The work was financially supported by the European Commission (BIO-4-CT-960419)

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4. Die Stimulation der CH_4-Oxidation durch NH_4^+-Düngung in der Reisrhizosphäre

In dieser Arbeit konnte gezeigt werden, dass NH_4^+-Düngung die methanotrophe Lebensgemeinschaft in der Reisrhizosphäre stimulierte und zu höheren CH_4-Oxidationsraten führte. Dies war ein überraschendes Ergebnis, da NH_4^+ in anderen Habitaten häufig die CH_4-Oxidation hemmte. Typ I und Typ II MOB wurden durch die höhere NH_4^+-Verfügbarkeit in Wachstum und Aktivität stimuliert, wobei Typ I MOB relativ stärker als Typ II MOB stimuliert wurden.

Die Populationsstruktur der Typ II MOB war im anoxischen Boden und in der Rhizosphäre gleich, und veränderte sich durch die NH_4^+-Düngungen nicht. Typ I MOB hingegen waren überwiegend in der Rhizosphäre vorhanden, wo CH_4-Oxidation durch den O_2-Eintrag über die Reiswurzeln ermöglicht wurde. Durch die NH_4^+-Düngungen wurde die Populationsstruktur der Typ I MOB leicht verändert.

Nature, Vol 403, 27 Januar 2000

Stimulation by ammonium-based fertilisers of methane oxidation in soil around rice roots

Paul L.E. Bodelier[*†], Peter Roslev[‡], Thilo Henckel[*] and Peter Frenzel[*]

[*]Max-Planck-Institute for terrestrial microbiology, Department of biogeochemistry Karl-von-Frischstrasse, D-35043 Marburg, Germany

[†] Centre for Limnology, Netherlands Institute of Ecology, Rijksstraatweg 6, 3631 AC, Nieuwersluis, The Netherlands

‡ Environmental Engineering Laboratory, Aalborg University, Sohngaardsholmsvej 57, DK-9000, Aalborg, Denmark

Methane is an important gas involved in key chemical and physical reactions in the earth's atmosphere including global warming[1]. Atmospheric methane originates mainly from biogenic sources such as rice paddies and natural wetlands. Rice paddies account for at least 30% of the global annual emission of methane to the atmosphere[2]. The estimated increase of the human population during the next three decades can only be sustained by an increase in rice production by 60%[3]. Since nitrogen supply drives rice productivity, intensified global fertiliser application will be inevitable[3]. An increase in the commonly used ammonium-based fertilisers can enhance methane emission from rice agriculture. Approximatly 10-30% of the methane produced by methanogens in rice paddies is consumed by methane-oxidising bacteria associated with the roots of rice[4,5]. The latter bacteria are generally thought to be inhibited by ammonium-based fertilisers as was demonstrated for soils[6-8] and sediments[9,10]. However, in this study we demonstrate a clear stimulation of activity and growth of methane-oxidising bacteria in the rhizosphere of rice plants following fertilisation. Using a combination of radioactive fingerprinting[11] and molecular biology[12] techniques the responsible bacteria for this surprising effect, with global climatic implications, were indentified.

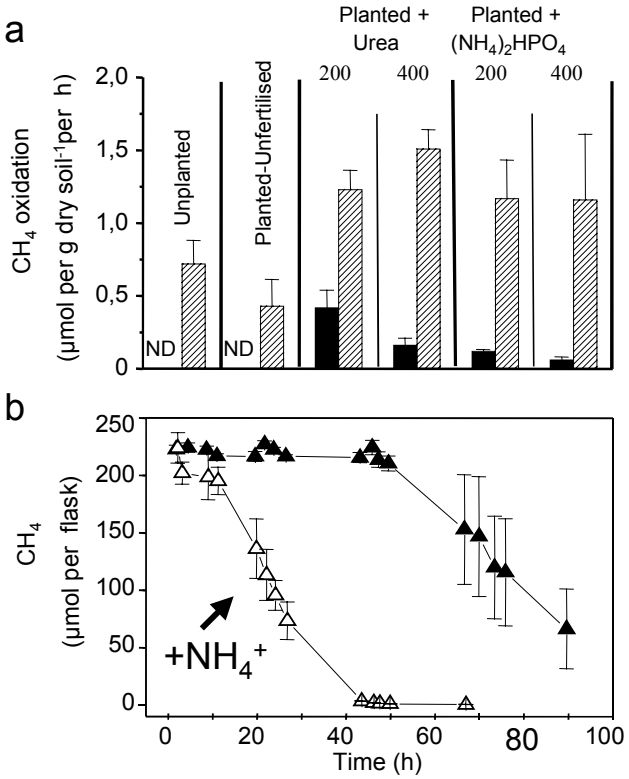

Figure 1. The effect of ammonium-based fertilisation and rice plants on methane oxidation rates of unplanted and rhizospheric soil from microcosms which were either unfertilised or supplemented with urea (200 or 400 kgN.ha^{-1}) or $(NH_4)_2HPO_4$ (200 or 400 kgN.ha^{-1}). The bars in panel A represent the arithmetric means of the initial- (Black bars) and induced oxidation rates (grey bars) of 4 replicate microcosms (± 1sd). ND marks assays without a detectable initial oxidation rate. Panel B shows the effect of ammonium addition on methane oxidation of rhizospheric soil slurries from unfertilised microcosms. Closed triangles represent the non-supplemented slurries while the open triangles depict the methane depletion in the slurries supplemented with ammonium (end concentration, 2 mM). All values represent the arithmetric means of 4 replicate microcosms (± 1sd).

Methanotrophic bacteria utilise methane as sole carbon and energy source and are subdivided in type I and II on the basis of phylogeny, physiology, morphology and biochemistry including characteristic phospholipid ester-linked fatty acids in their cellmembranes[13]. These obligatory aerobic bacteria live in close association with the roots of wetland plants (e.g. rice) which provide them with necessary oxygen[14,15] to oxidise methane which diffuses from the anoxic bulk soil to the rhizosphere. In the present study the effect of fertilisation on methane oxidation was examined using microcosms planted with rice with a root- and a bulk soil compartment. The application of urea or diammoniumphophate (200 or 400 kg N.ha^{-1}) stimulated rahter than inhibted methane oxidising activity in the root zone of rice (Figure 1A). All samples from planted, fertilised microcosms displayed initial methane-oxidising activity whereas no initial activity (0-12h) could be detected in soil samples from the rhizosphere of the unfertilised and the unplanted microcosms (Figure 1A). The induced activities were also significantly increased by fertilisation (Kruskall Wallis test, $p<0.001$) and the presence of rice plants (Kruskall Wallis test, $p<0.05$) (Figure 1A). With

the unfertilised samples it took 50 hours before any methane depletion could be detected (Figure 1B, closed triangles). However, ammonium addition to parallel soil slurries from unfertilised microcosms led to an immediate activation of the methane-oxidising bacteria (Figure 1B, open triangles), demonstrating the essential role of ammonium in the consumption of methane in rice soil.

The stimulating effect of fertilisation was also demonstrated in experiments with larger (1.5 l of soil), non-compartmented microcosms cultivated in a greenhouse. Methane oxidation increased substantially after urea application which was detected both by *in situ* rate measurements and as an increased $^{13}C/^{12}C$ ratio (less negative $\delta^{13}C$ value) of the emitted CH_4 (M. Krüger, P. Claus & P.Frenzel, in preparation).

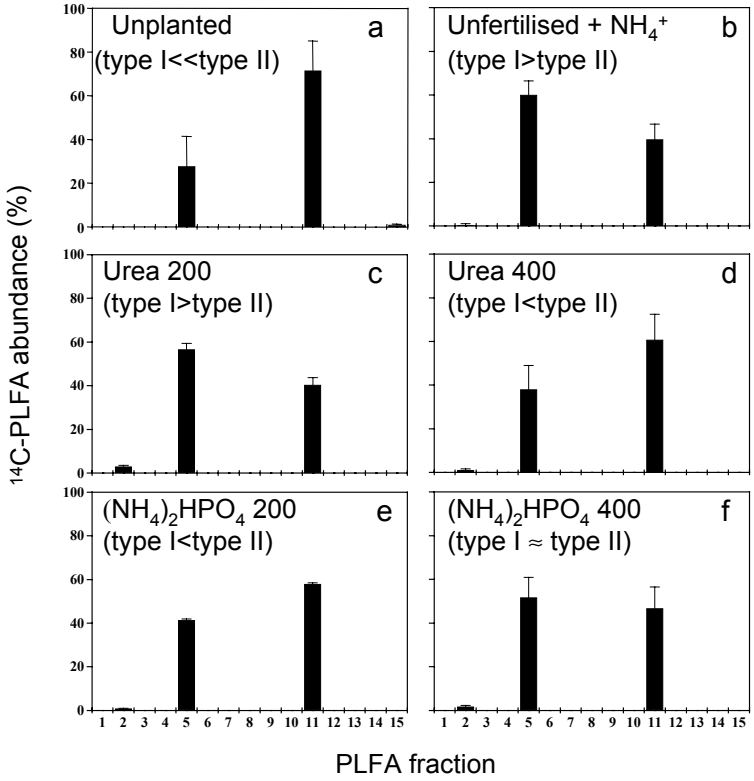

Figure 2. ^{14}C-labelled phospholipid ester-linked fatty acid (^{14}C-PLFA) fingerprints of methane-oxidising bacteria from soil samples incubated with $^{14}CH_4$. The fingerprints were obtained from unplanted (A), and the rhizosphere compartments from planted microcosms which were supplemented with urea 200 (C) or 400 (D) kgN.ha^{-1} or $(NH_4)_2HPO_4$ 200 (E) or 400 (F) kgN.ha^{-1}. Panel B displays the fingerprint from soil originating from the rhizosphere compartment of unfertilised microcosms which was supplemented with ammonium (compare Fig. 1B) in post-harvest slurry incubations. The graphs show the percentage of radioactivity detected in each PLFA fraction. $^{14}CH_4$ incorporation into PLFA which eluted mainly in fraction 5 (e.g.,16:1ω8, 16:1ω7, 16:1ω5 and 16:0) indicate methane oxidation by type I methane oxidisers while radioactive PLFAs detected mainly in fraction 11 (e.g.,18:1ω9, 18:1ω8, 18:1ω7, and 18:0) indicate oxidation dominated by type II methane oxidisers. Bars indicate the mean (± 1sd) of 2 subsamples taken from 1 out of 4 replicate microcosms per treatment. The fingerprint in the „unfertilised" panel corresponds to the ammonium supplemented slurry shown in figure 1. The metabolism of $^{14}CH_4$ was defined as dominated by type I methane-oxidising bacteria when the ratio fraction 5: fraction 11 was greater than 1.1. If this ratio was lower than 1.1, type II methanotrophs was assumed to dominate. This differentiation is based on laboratory experiments with pure cultures of type I and II methane-oxidising bacteria[27].

The bacteria responsible for the rather uncommon observations were indentified using radioactive fingerprinting and molecular biology techniques. Figure 2 shows that in all treatments radiolabelled methane was incorporated into PLFA fractions that contained fatty acids typical of type I and II methane-oxidising bacteria . Type Il methane oxidisers dominated methane metabolism in unplanted, unfertilised soil as indicated by recovery of ^{14}C-PLFAs in fraction 11 mainly (Figure 2A). In contrast, long term-fertilisation of planted soil with either urea or ammonium diphosphate resulted in the activity of both type I and II methane oxidisers in the rhizosphere (Figure 2 B-F). The apparent activation of type I methane-oxidising bacteria by fertilisation was confirmed by the ^{14}C-PLFA fingerprints obtained after addition of ammonium to soil slurries retreived from the rhizosphere (Figure 2B). More radioactivity was recovered in PLFA fraction 5 after addition of ammonium to such samples from unfertilised microcosms (Figure 2B).

The total abundance of the PLFAs specific for methanotrophic bacteria (16:1ω8c for type I and 18:1ω8c for type II), can be regarded as a measure of methanotrophic biomass[16]. Our data revealed that fertiliser addition to planted microcosms resulted in a 2-3 times increase in the abundance of PLFAs specific for methanotrophic bacteria (Figure 3). This was supported by using a most probable number approach where higher (Kruskall-Wallis test, $p<0.001$) numbers of culturable methanotrophs were found in fertilised microcosms[17]. Fertilisation resulted in growth of type I methane oxidisers mainly as indicated by a 9 times increase in the type I specific PLFA biomarker in microcosms fertilised with 400 $kgN.ha^{-1}$ of urea or $(NH_4)_2HPO_4$ (Figure 3). The type II specific PLFA 18:1ω8c increased only by a factor 2-3 after fertilisation, however, this PLFA remained the dominant methanotrophic biomarker in fertilised samples. The dominance was most pronounced in unplanted and unfertilised samples where the type II specific biomarker was 7 times more abundant than type I biomarkers (Figure 3).

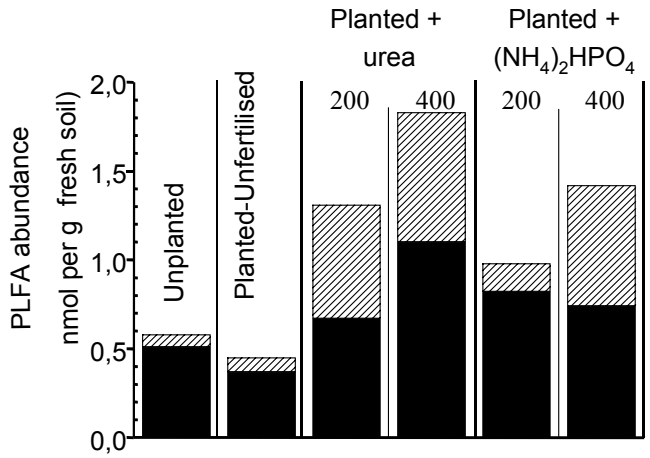

Figure 3 Total abundance of the type I specific PLFA 16:1 ω8c (hatched bars) and the type II specific PLFA 18:1 ω8c (black bars) in samples retrieved from unplanted soil and from the rhizosphere compartments of planted microcosms which were either unfertilised or supplemented with urea (200 or 400 kgN.ha^{-1}) or $(NH_4)_2HPO_4$ (200 or 400 kgN.ha^{-1}).

By combining the PLFA profiles and DGGE separation of pcr-amplified 16S rDNA, a higher phylogenetic resolution of the methane-oxidising community in rice soil was obtained. The DGGE banding patterns (Figure 4) and the respective sequences (Figure 5) demonstrate that type and dose of fertiliser had little effect on the species composition of the methane-oxidising community. Type I bands (RRI 1-RRI 6, RBI 1) were most closely related to *Methylobacter* species (Figure 5) while type II bands (RRII 3- RRII 7) were related to *Methylosinus* and *Methylocystis* species. All major bands, methanotrophic as well as non-methanotrophic, were highly related to sequences and clones from rice field soil incubations[12] (Figure 5). DGGE bands related to type II methane oxidisers were equally intense in all samples while type I-related DGGE bands were distinctly more intense in the rhizosphere samples (Figure 4). Henckel et al[12] showed that these intensity differences are the consequence of differences in original number of target sequences and hence on the numbers of the respective methane oxidisers in the soil. Thus, next to the ammonium the presence of the rice plant is an essential factor for type I methane-oxidising bacteria to proliferate. This finding is supported by the PLFA data.

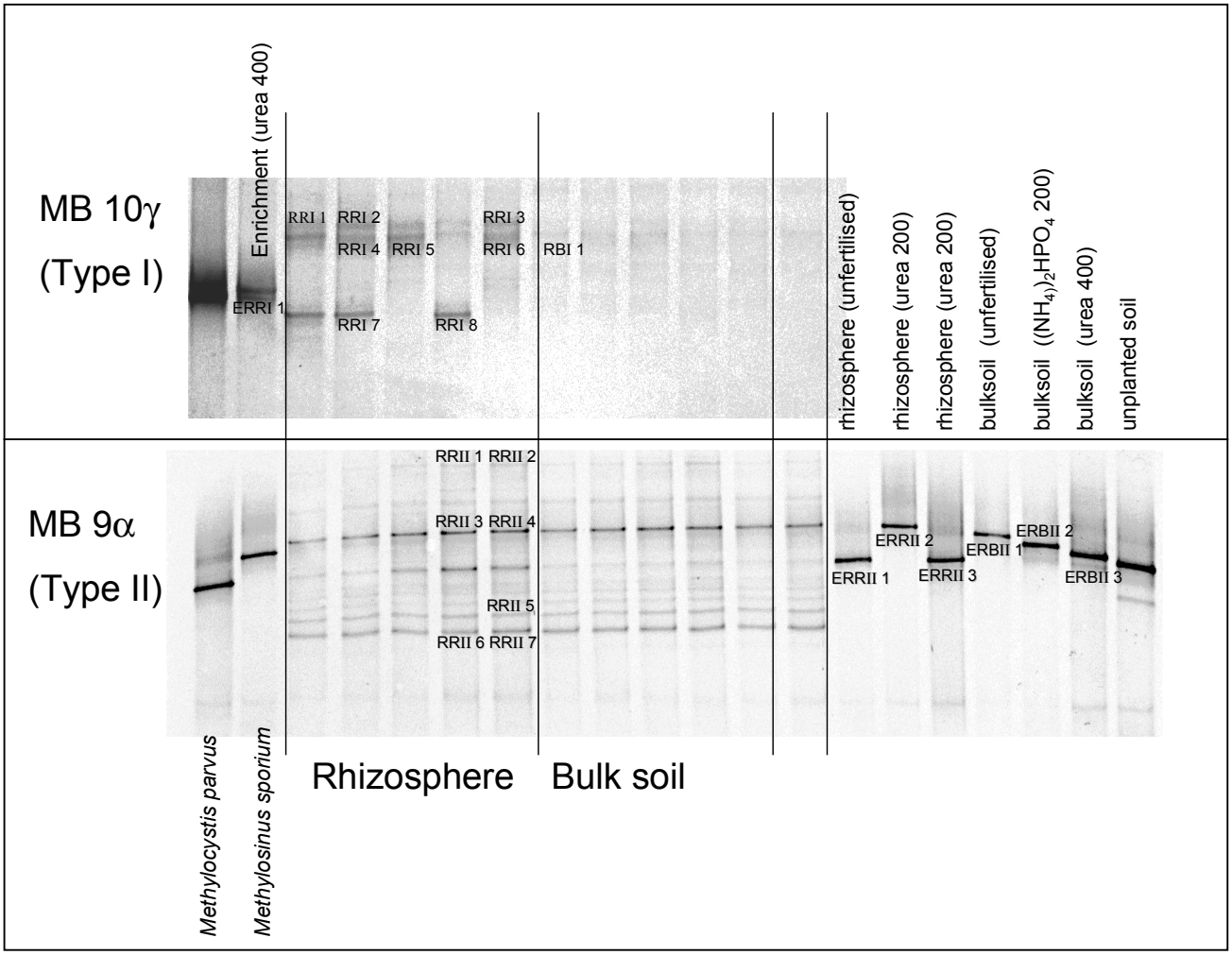

Figure 4 Effect of the presence of rice plants and fertilisation on the composition of the methane-oxidising community in rice soil microcosms. Displayed are DGGE (denaturing gradient gel electrophoresis) banding patterns obtained after amplifying DNA extracted from soil and enrichment cultures from the highest positive MPN dilutions with an MB10γ (upper panel) 16S rDNA primer set targeting type I methanotrophs and obtained with an MB9α (lower panel) 16S rDNA primer set targeting type II methanotrophs. The samples were retreived from unplanted soil and from the rhizosphere and bulk soil compartments of planted microcosms which were either unfertilised or supplemented with urea (200 or 400 kgN.ha^{-1}) or $(NH_4)_2HPO_4$ (200 or 400 kgN.ha^{-1}). The DGGE patterns of the rhizosphere and unplanted samples correspond to the respective PLFA patterns in figure 2. Bands designated with acronyms were excised from the gels, reamplified and sequenced as described[12]. The phylogenetic positions of the derived sequences are shown in figure 4.

Enrichments from the highest positive dilutions of MPN counts contained only type II methanotrophs (Figure 4, lower panel). These bands (ERRII 1-3, ERBII 1-3) matched the dominant bands obtained from soil samples, confirming the numerical dominance of type II methanotrophs in rice soil that was indicated by the PLFA analysis (Figure 3).

This study clearly demonstrates that ammonium-based fertilisation does not necessarily inhibit the consumption of methane in soils and sediments as has been repeatedly reported[6-10]. The absence of inhibition may be explained by the high methane availability[17] in rice paddies which counterbalances possible competitive

inhibition by ammonium[18]. Furthermore fertiliser associated nitrite toxicity[7] or salt effects[19,20] on methane oxidation may be eliminated by plant uptake of these ions. Such a sink for toxic substances is missing in upland soils where also methane concentrations are low, explaining the reported inhibition of methane oxidation by ammonium in soils and soil incubations[6-10].

Both type I and II conventional methanotrophs need ammonium to be active and to grow in the rice rhizosphere. However, the number of type I methane oxidisers was elevated to a greater extend relative to the number of type II methane-oxidising bacteria upon fertilisation. N availability has been suggested to select for type I methane oxidisers[13] but experimental evidence was missing.

Our data suggest that fertiliser application may lead to a reduction rather than to an elevation of methane emission from wetland rice fields. Methane emission from the microcosms used in this study was reduced up to 57% by fertiliser application[17]. Moreover, a significant negative correlation was found between porewater ammonium concentrations and methane emission, indicating elevated methane oxidation to be the reason for this reduction[17]. However, we[17] and other investigators[21,22] demonstrated that fertilisation can also enhance methane production due to higher plant biomass and subsequent carbon availibility for methanogens. The net emission will be the balance between enhanced consumption and production. Our study will therefore lead to reevaluation of fertiliser practice and studies on methane emission from soils and sediments .

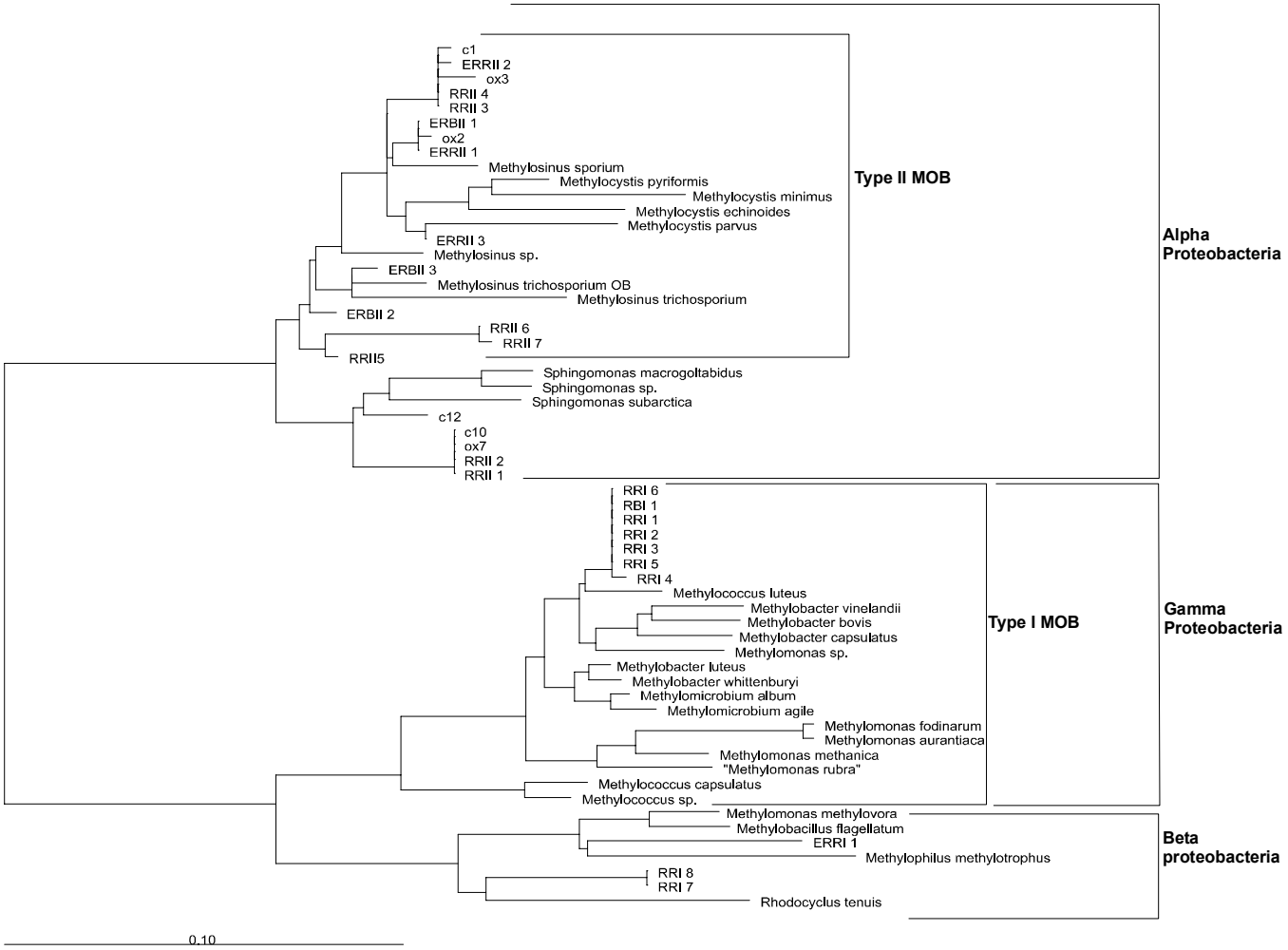

Figure 5 Phylogenetic tree showing the relationship of partial 16S rDNA sequences retrieved from DGGE gels (see figure 3) with the most closely related members of the α, β and γ proteobacteria. The scale bar indicates the estimated number of base changes per nucleotide sequence position. "c" and "ox" are clones from rice field soil incubated without and with methane, respectively[12].

Methods

Microcosms. Experimental system and design have already been described[17,23]. The microcosms had a physical separation between rhizosphere and bulk soil and were incubated in growth cabinets for a period of 90 days at 70% RH and in a light/dark cycle of 12/12 hours at a photosynthetically active radiation (PAR) of 450 µEinstein·m^{-2}·s^{-1} (99 W.m^{-2}) and a temperature regime of night/day of 20°C/25°C. The surface of the soil was always covered with 2 cm of demineralised water. Soil and seeds (*Oryza sativa* var. Roma, type japonica) originated form a Italian rice paddy (Vercelli, Nothern Italy).The microcosms were either unplanted, planted and unfertilised or planted and supplemented with urea (200 or 400 kgN.ha^{-1}) or $(NH_4)_2HPO_4$ (200 or 400 kgN.ha^{-1}). The fertiliser was added twice per week by injection into the soil with a syringe. After 90 days of incubation the soil from the rhizosphere and bulk soil compartments was harvested and soil slurries were prepared[24] for performing the analyses described below.

Methane oxidation rates. Methane oxidation rates (Figure 1A) were determined by transferring subsamples of the slurries described above to flasks which were supplemented with CH_4 (10 000 ppmv). CH_4 depletion was monitored by GC analysis of the headspace gas as described earlier[24]. From the sigmoidal CH_4 depletion curves (see figure 1B, open triangles) initial (0-12 hours) and induced (12-24 hours) oxidation rates were calculated by linear regression. To demonstrate the nitrogen-limitation of methane oxidation in unfertilised microcosms, slurries from unfertilised microcosms were supplemented with $(NH_4)_2SO_4$ (final concentration 2 mM N).

^{14}C-PLFA profiles. Directly after harvesting of the soil and preparation of soil slurries, 20 ml of slurry was transferred to a polyethersulfon membrane (pore size 0.2 µm, diameter 47 mm) by vacuum filtration. The membranes (with about 4 g of fresh soil) were transferred to 150-ml flasks, supplemented with 0.15 MBq $^{14}CH_4$ and incubated at 25°C for 3 days. PLFAs were extracted as described elsewhere[11]. Radiolabelled PLFAs were separated into 15 fractions by capillary GC and collected as $^{14}CO_2$ after combustion in the flame ionization detector. The radioactivity in each fraction was determined by liquid cintillation counting.

PCR and DGGE of soil DNA. DNA was extracted from soil and amplified by PCR as already described[12] . The primer sets MB10γ and MB9α amplify 16S rDNA of methylotrophic bacteria belonging to the γ- and α proteobacteria[12], respectively. PCR products were separated by DGGE using a 35-70% and 45-70% denaturant gradient for MB10γ and MBα PCR products, respectively. For pure cultures and enrichments, 10 µl of pcr product was loaded onto the gel, while for soil samples 45 µl was applied. Bands were excised from the gels, reamplified and sequenced as described[12]. The phylogenetic position of the derived sequences are shown in figure 4.

Phylogeny. 16S rDNA sequences were aligned and placed phylogenetically with the ARB software package[25]. Evolutionary distances between pairs of sequences were calculated by using the Jukes-Cantor and Felsenstein algorithms in the ARB software. The phylogenetic trees were constructed using the neighbour-joining algorithm supplied with the ARB package, based on complete 16S rDNA sequences of α, β and γ proteobacteria. The partial 16S rDNA sequences retrieved from DGGE gels were added to the tree by keeping its topology constant[26]. Sequences of partial 16S rRNA gene fragments of excised DGGE bands have been deposited in the GenBank database under accession nos AF179599-AF179610.

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Acknowledgements

The authors would like to thank Prof. Ralf Conrad and Dr. Peter Dunfield for the critical reading of the manuscript, Dr. Michael Friedrich for supporting the molecular studies and Bianca Wagner and Sonja Fleisner for their excellent technical assistance. The project was financially supported by the EU, the Danish Technical Research Council and the DFG, (Deutsche Forschungs Gemeinschaft).

Correspondence and request for materials should be addressed to PLE Bodelier at the Centre for Limnology, Netherlands Institute of Ecology, Rijksstraatweg 6, 3631 AC Nieuwersluis, The Netherlands. (email: bodelier@cl.nioo.knaw.nl).

5. Die Charakterisierung einer unbekannten methanotrophen Lebensgemeinschaft in Waldboden mit atmosphärischer CH_4-Oxidation

Waldböden sind wichtige Senken für CH_4 aus der Atmosphäre. Atmosphärisches CH_4 wurde in dem hier untersuchten sauren Waldboden aufgenommen und bis zu einer Tiefe von 26 cm oxidiert. Über das *pmo*A-Gen der partikulären Methan-Monooxygenase wurde eine Lebensgemeinschaft unbekannter MOB nachgewiesen und charakterisiert. Die *pmo*A-Sequenzen waren nur entfernt mit den *pmo*A-Sequenzen bekannter MOB verwandt und stellen wahrscheinlich eine neue Gruppe methanotropher Bakterien dar. Atmosphärisches CH_4 im Waldboden wird daher möglicherweise durch diese unbekannten MOB oxidiert.

Applied Environmental Microbiology, 2000, in press.

Molecular analyses of novel methanotrophic communities in forest soil oxidizing atmospheric methane

Thilo Henckel, Udo Jäckel, Sylvia Schnell and Ralf Conrad*

Max-Planck-Institut für terrestrische Mikrobiologie, Karl-von-Frisch-Strasse, 35043 Marburg, Germany

Abstract

Forest and other upland soils are important sinks for atmospheric CH_4 consuming 20-60 Tg CH_4 per year. Consumption of atmospheric CH_4 in soil is a microbiological process. However, little is known about the methanotrophic bacterial community in forest soils. We measured vertical profiles of atmospheric CH_4 oxidation rates in a German forest soil and characterized the methanotrophic populations by polymerase-chain-reaction (PCR) and denaturing-gradient-gel-electrophoresis (DGGE) with primer sets targeting the *pmo*A gene, coding for the α-subunit of the particulate methane-monooxygenase (pMMO), and the small subunit ribosomal RNA gene (SSU rDNA) of all life. The forest soil was a sink for atmospheric CH_4 in situ and in vitro at all times. In winter, atmospheric CH_4 was oxidized in a well defined subsurface soil layer (6 cm to 14 cm soil depth), whereas in summer the complete soil core was active (0 cm to 26 cm soil depth). The content of total extractable DNA was about tenfold higher in summer than in winter. It decreased with soil depth (0-28 cm depth) from about 40 to 1 µg DNA per gram dry weight (gdw) soil. The PCR product concentration of SSU rDNA of all life was constant, both in winter and summer. However, the PCR product concentration of *pmo*A changed with depth and season. *Pmo*A was only detected in soil layers with active CH_4 oxidation, i.e. in 6-16 cm in winter and throughout the soil core in summer. The same methanotrophic populations were present in winter and summer. Layers with high CH_4 consumption rates also exhibited more DGGE bands of *pmo*A, indicating that high CH_4 oxidation activity was positively correlated to the number of methanotrophic populations present. The *pmo*A sequences derived from excised DGGE bands were only distantly related to known methanotrophs, indicating the existence of unknown methanotrophs involved in atmospheric CH_4 consumption.

Introduction

The atmospheric concentration of CH_4, one the most important greenhouse gases, has increased dramatically over the past 200 years. About 80-90% of the atmospheric CH_4

is of biogenic origin (21). The major sink is the chemical destruction by OH- and Cl-radicals in the troposphere and stratosphere, respectively (9, 10). However, the capacity of these atmospheric sinks may decline, since the rising concentrations of other trace gases emitted by anthropogenic activity, result in a reduction of OH-radicals in the troposphere (28).

The only biological sink for CH_4 is the oxidation in soil. Atmospheric CH_4 is consumed in forest, agricultural and other upland soils. Consumption in these soils is caused by methane-oxidizing bacteria. However, the identity of these methanotrophs is still unknown. The apparent half saturation constants (K_m) for oxidation of atmospheric CH_4 (approximately 1.8 ppmv) in upland soils range from 0.8 to 280 nM (6, 7, 13, 17). However, the K_m of the common type I or II methanotrophs (0.8 to 66 µM), that are available in culture collections, is 1 to 3 orders of magnitude higher and these common methanotrophs are not able to survive for a prolonged period of time using only atmospheric CH_4 (14, 39, 18). Recently however, a type II methanotroph was isolated from a humisol able to adapt to nanomolar K_m values, close to those measured in upland soils (14,15). The existence of this isolate questioned the hypothesis of Bender & Conrad (1992) that besides the common low-affinity methanotrophs (micromolar K_m), unknown high-affinity methanotrophs (nanomolar K_m) exist, and that only the latter are responsible for atmospheric CH_4 oxidation (6).

Common methanotrophs have a neutral pH optimum (19). However, most forest soils are slightly acidic around pH 5 and lower. Bacteria extracted from forest soils showed a methanotrophy pH optimum of 5.8, indicating that unknown acidophilic methanotrophs may be responsible for CH_4 oxidation in forest soils (5). Indeed, an acidophilic methanotroph, belonging to the *α-Proteobacteria* and closely related to the non-methanotroph *Bejerinckia* but only distantly related to the common type II methanotrophs, was recently isolated from an acidic blanket peat bog in Russia (11, 12).

However, so far little is known about the methanotrophic community in forest soil. A recent analysis of *pmoA* gene libraries from forest soils has demonstrated the existence of a new group of methanotrophs in various forest soils (24). These soils all exhibited uptake of atmospheric CH_4.

Forest soils have extensively been studied with respect to CH_4 oxidation kinetics, zonation and inhibition of CH_4 oxidation, because of their important function as major sinks in the global CH_4 budget. The highest CH_4 oxidation activity in forest soils was usually measured in subsurface soil layers (1, 30, 35, 41). This localization of methanotrophs in deeper soil layers was attributed to inhibition of methanotrophs by ammonium or terpenes that are released or produced in the organic surface layers of the forest soil (2, 3, 4, 17, 27, 38). However, the zonation of CH_4 oxidation in relation to the involved methanotrophic community has not yet been studied.

Therefore, we measured vertical profiles of the uptake of atmospheric CH_4 in forest soil cores in winter and summer, and characterized the bacterial and involved methanotrophic populations by PCR-DGGE using a universal SSU rDNA primer set and a primer set targeting the *pmoA* gene.

Materials and Method

Sampling site and soil characteristics. The sampling site was located on a slope in a deciduous forest near Marburg, Germany (N 51°00´; E 09° 50´), consisting of mainly beech (*Fagus sylvatica*) and oak trees (*Quercus robur*). The soil type was a cambisol with a A_h (2 - 6 cm), B_v (6 - 28 cm), and C (sandstone) horizon. The soil originated on sandstone and was a loamy sand. The pH_{H2O} of the organic A_h horizon and the mineral subsoil was pH 3.8 and pH 4.3, respectively. Soil cores were collected in January (winter) and July (summer) of 1999.

The maximal water holding capacity (WHC) was determined for the organic soil (A_h) and the mineral subsoil (B_v) according to Schlichting & Blume (1960), and was 0.46 ± 0.04 and 0.39 ± 0.02 g H_2O gdw^{-1}soil (n = 5), respectively (37). The gravimetric water content was determined for each 2-cm vertical soil section and expressed as %WHC.

Vertical CH_4 concentration profiles. Gas samples were taken by pushing a PEEK-capillary (∅ 0.35 mm; Sykam, Gilchingen, Germany) attached to a stainless steal rod in 1-cm intervals into the soil. Prior to sampling the tube was flushed by extracting 0.1 ml gas. Gas samples (1 ml) were collected in gas tight syringes and analyzed by gas chromatography (GC) with a GC-8A (Shimadzu, Japan) equipped with a flame ionization detector and a stainless steel column (2 m, ∅ 1/8 inch) filled with Poropak Q (mesh 80/110).

Collection of soil cores and in vitro CH_4 oxidation rate. Soil cores were taken with Plexiglas corers (∅ 6 cm) pushed into the forest floor. The columns were extracted from the ground and closed with silicone stoppers. In the laboratory, the cores were gently pushed upward and cut into 2-cm sections. The sectioned soil layers were immediately transferred into 150-ml serum bottles and closed with latex stoppers.

Gas samples were repeatedly taken over time with gas-tight pressure-lock syringes (Dynatech A-2 Series, Baton Rouge, La, USA) and analyzed by GC. Apparent first order CH_4 oxidation rate constants were calculated (Excel 7.0, Micosoft) from the exponential decrease of CH_4 with time and converted to CH_4 oxidation rates by multiplication with the atmospheric CH_4 mixing ratio (1.7 ppmv).

DNA extraction. DNA extraction from forest soil was modified after Moré et al. (33). Approximately 0.5 g (fresh weight) soil was taken from the each forest soil layer (2 cm thick) and transferred into 2-ml screw cap tubes. Approximately 1 g of sterilized (170°C for

4 h) zirconia/silica beads (0.1 mm diameter; Biospec products Inc., Bartlesville, Ok, USA), 800 µl Na-phosphate buffer (120 mM, pH 8), and 260 µl SDS-solution (10% SDS, 0.5 M Tris/HCl, pH 8.0, 0.1 M NaCl) were added, and the soil resuspended homogeneously by vortexing. Cells were lysed for 45 s by shaking in a cell disruptor (FP120 FastPrep, Savant instruments Inc., Farmingdale, NY, USA) at a setting of 6.5 m s^{-1}. After centrifugation (3 min at 12,000 × g) the supernatant was collected, and the soil-beads mixture was extracted a second time by resuspension in 700 µl phosphate buffer. Protein and debris were precipitated from the supernatant by adding 0.4 volumes of 7.5 M ammonium acetate, followed by incubation on ice for 5 min. After centrifugation at 12,000 × g for 3 min nucleic acids were precipitated by addition of 0.7 volumes of isopropanol, followed by centrifugation at 12,000 × g and 4°C for 45 min. Subsequently, the DNA pellet was washed with 70% ethanol at 4°C, and dried under vacuum. Finally, DNA was resuspended in 200 µl Tris-EDTA buffer (10 mM Tris-base, 1 mM EDTA, pH 8).

Removal of humic acids. The forest soil DNA extracts were of a dark brown color and contained large amounts of humic acids. The humic acids were removed with acid-washed polyvinyl-polypyrrolidone (PVPP) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in Spin columns (BioRad, Munich, Germany) modified after Holben et al. (22). The spin columns were filled with 2 ml PVPP, which had been equilibrated and suspended in Tris-EDTA pH 8. The PVPP columns were packed and dried by centrifugation (375 x g, 1 min) just prior to loading. About 150 µl of the brown humic acid-containing soil DNA extract was loaded onto the column and centrifuged. The purified DNA solution was clear and colorless, and readily amplifiable by PCR.

Concentration and purity of the DNA solutions were determined by absorption at 260 nm and 280 nm after 1:10 dilution in H_2O using a GeneQuant spectrophotometer (Pharmacia Biotech, Upsala, Sweden). For PCR amplification, DNA aliquots at a standardized DNA concentration of 1 ng μl^{-1} were used.

PCR amplification. For PCR amplification we used a "universal" SSU rRNA-based primer set, targeting all life and the functional primer set "pmoA" (20).

PCR buffer (20 mM Tris-HCl, pH 8.3, 50 mM KCl), 1 U and 0.5 U AmpliTaq DNA polymerase for the pmoA and the Universal primer set, respectively (Perkin Elmer Applied Biosystems, Weiterstadt, Germany), 0.5 µM of each primer, 100 µM of each deoxynucleoside triphosphate (Amersham Life Science, Braunschweig, Germany) were added to a total reaction volume of 50 µl at 4°C. For the *pmoA* amplification the MasterAmp 2×PCR premix F containing 100 mM Tris-HCl (pH 8.3), $MgCl_2$, 400 µM of each deoxynucleoside triphosphate, and the PCR enhancer betaine (Epicentre Technologies, Madison, WI, USA) were

were added to the reaction solutions. The same template concentration (1 to 5 ng μl^{-1}) was always used in a set of PCR amplifications from the same soil core. Amplifications were started by placing cooled (+4°C) PCR tubes immediately into the preheated (94°C) thermal block of a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). The thermal cycling profiles consisted of touchdown programs with an initial denaturation of 3 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at the annealing temperature, and 45 s at 72°C, and 5 min at 72°C for the last cycle. The annealing temperature decreased from 62°C to 55°C and from 60°C to 50°C in 0.5°C steps for the pmoA and Universal primer sets, respectively.

Aliquots (5 µl) of PCR products were analyzed by electrophoresis on 3% agarose gels, stained with ethidium bromide, and quantified densitometrically. Gels were destained in water for 30 min. For calibration the Smart-Ladder DNA mass and size ruler (Eurogentec, Seraing, Belgium) was used (calibration coefficient of all analyses, $r > 0.9$). Gels were photographed with an imaging system (MWG Biotech, Germany), and DNA bands were analyzed with the RFLP-scan software (CSP Inc., Billerica, Belgium).

DGGE. DGGE was carried out as described previously in detail (20Henckel et al., 1999). PCR products were separated using a DCode System (Bio-Rad, Munich, Germany) on 1-mm thick polyacrylamide gels (6.5% w/v acrylamide:bis acrylamide (37.5:1); Bio-Rad) prepared with and electrophoresed in 0.5 × TAE, pH 7.4 (0.04 M Tris-base, 0.02 M sodium-acetate, 1 mM EDTA) at 60°C, and constant voltage. A denaturing gradient of 35-80% and 35-70% was used for the pmoA and Universal PCR products, respectively. A denaturing gradient of 80% (vol/vol) denaturant corresponded to 6.5% acrylamide, 5.6 M urea and 32% deionized formamide. Gels were poured onto GelBond PAG film (FMC Bioproducts, Rockland, ME, USA) to avoid gel distortion. Gels were stained with 1:50,000 (vol/vol) SYBR-Green I (Biozym, Hessisch-Oldendorf, Germany) for 30 min, and scanned with a Storm 860 phosphor imager (Molecular Dynamics, Sunnyvale, CA, USA). The scanned DGGE gels were digitally enhanced with Adobe-Photoshop 5.0 (Adobe Systems Incorporated) to improve graphic resolution of the figures.

For further analysis, DGGE bands were visualized in the SYBR Green I-stained gels with blue light ($\lambda > 400$ nm) using a Dark Reader transilluminator (Clare Chemical Research, Ross on Wye, UK). Individual DGGE bands were then excised, reamplified, and reanalyzed by DGGE to verify band purity, as described recently (20).

Sequencing of DGGE bands. Reamplified PCR products of excised DGGE bands were purified using the EasyPure DNA purification kit (Biozym, Hessisch-Oldendorf, Germany). Concentration and purity of PCR products were determined by absorption at 260 nm and 280 nm of a 1:20 dilution in H_2O with a GeneQuant spectrophotometer (Pharmacia Bio-

tech, Upsala, Sweden). Sequencing reactions were performed using the ABI Dye-terminator cycle sequencing kit (Perkin Elmer Applied Biosystems,) as specified by the manufacturer. Cycle sequencing products were purified from excess dye terminators and primers using Microspin G-50 columns (Pharmacia Biotech, Freiburg, Germany), and analyzed with an ABI 373 DNA sequencer (Perkin Elmer Applied Biosystems).

Sequences were analyzed using the Lasergene software package (DNASTAR, Madison, WI, USA). Nucleotide and inferred amino acid sequences of the gene fragments of *pmo*A were manually aligned with sequences retrieved from the GenBank database. SSU rDNA sequences were aligned and phylogenetically placed with the ARB software package (40). The partial SSU rDNA sequences were added to a validated and optimized tree of about 5000 16S rDNA sequences with more than 1400 residues each, while keeping the overall topology constant (31). On the nucleic acid level, evolutionary distances between pairs of sequences were calculated by using the Jukes-Cantor, and Felsenstein equations (16, 26) implemented in the ARB package. Phylogenetic trees were constructed by using distance matrix and maximum parsimony methods supplied by the ARB software package (40).

Nucleotide accession numbers. The sequences of *pmo*A gene fragments and of the SSU gene fragments of excised DGGE bands have been deposited in GenBank under accession numbers AF200726 to AF200729, and AF200730 to AF200734, respectively.

Results

CH_4 consumption rates of forest soil. In situ CH_4 mixing ratios decreased with soil depth both in winter and summer (Fig. 1). In winter, the CH_4 mixing ratios stayed constant in the upper 2 cm soil layers at ambient values, and showed a linear decrease between 3 to 8 cm soil depth (Fig. 1A). Below 8 cm soil depth CH_4 decreased rapidly, indicating CH_4 consumption (Fig. 1A). In summer, the in situ profile showed a CH_4 decrease from 0 to 16 cm depth (Fig. 1B). Below 16 cm depth, the in situ CH_4 mixing ratios remained constant at approximately 0.25 ppmv CH_4. The forest soil was always a net sink for atmospheric CH_4, exhibiting a CH_4 consumption rate of 1.00 ± 0.26 mg $m^{-2}d^{-1}$ (mean ± SD of n = 3) in winter. Similar values were reported before (1, 30, 41).

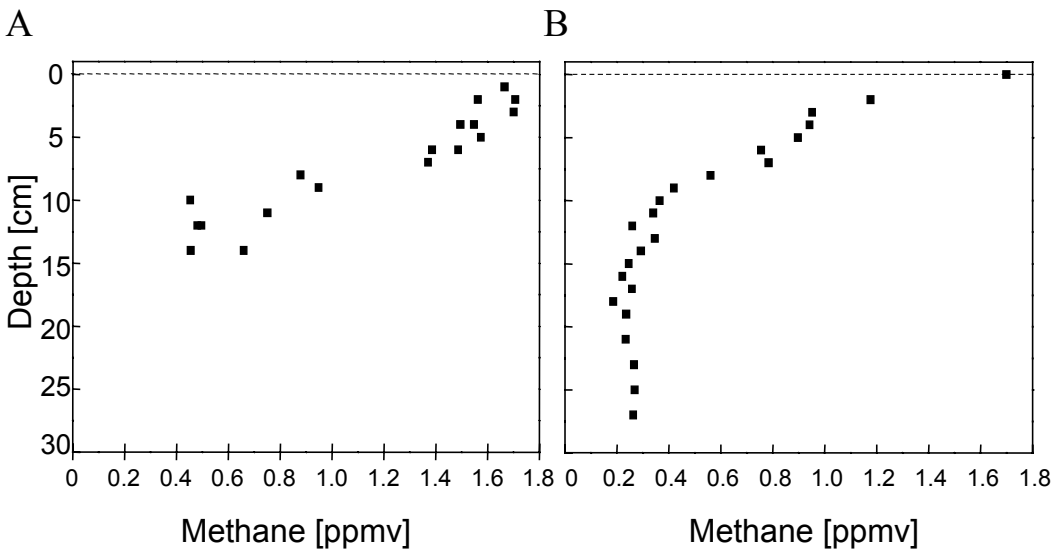

Figure 1. Vertical profiles of in situ CH_4 mixing ratios in forest soil (A) in winter (January, 1999) and (B) in summer (July, 1999).

The in vitro CH_4 oxidation rates were measured in distinct 2-cm soil sections between 0 cm and 16 cm depth in winter, and 0 and 28 cm depth in summer (Fig. 2A). In winter, no CH_4 consumption was detected between 0 and 6 cm soil depth. CH_4 oxidation started at 6 cm depth and maximal oxidation rates were measured between 10 and 14 cm depth. The oxidation rates sharply decreased below 14 cm depth. In summer, on the other hand, CH_4 oxidation activity was measured between 0 and 26 cm soil depth. Maximal oxidation rates were measured between 4 and 10 cm depth, below 12 cm the CH_4 oxidation rates decreased sharply and ceased below 26 cm (Fig. 2A).

The water content of the loamy sand decreased with depth. In winter, the water content exceeded 100% WHC between 0 and 10 cm depth and dropped to about 50% WHC below 12 cm depth. In summer, the forest soil exhibited a water content of approximately 70% WHC at the surface (0 - 2 cm), decreasing to approximately 13% WHC below 26 cm depth (Fig. 2B).

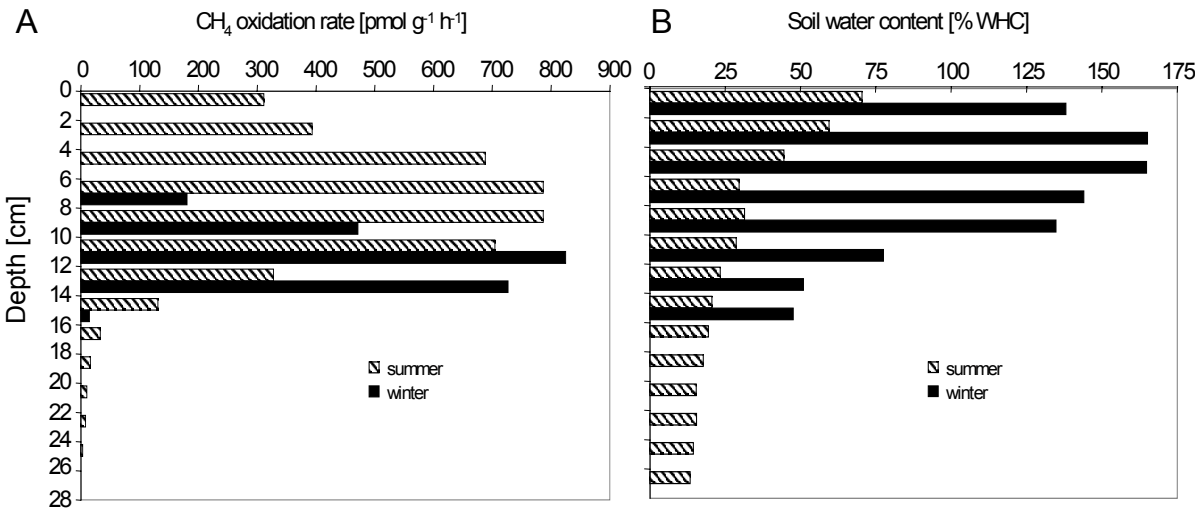

Figure 2. Vertical profiles of (A) CH_4 oxidation rates per gram fresh soil, and (B) soil water content, both in winter (black bars) and summer (hatched bars).

DNA extraction and PCR amplification. In summer, up to 10 times more total DNA was extracted from the forest soil than in winter (Fig. 3A). The total extractable DNA content of the soil decreased with depth, both in winter and summer. In summer, the decrease of total extractable DNA was most pronounced (Fig. 3A).

PCR with the universal primer set, targeting the SSU rDNA of all life, amplified PCR products from all depth layers in winter (25.7± 4.9 ng μl^{-1}; mean ± SD of n = 8) and summer (29.5 ±11.5 ng μl^{-1}; mean ± SD of n = 13) at similar concentrations, indicating that DNA from all soil layers was readily amplifiable and not subjected to different biases caused by humic acids or other PCR-inhibiting substances.

In contrast to the universal primer set, PCR with the primer set pmoA yielded product concentrations depending on soil depth and season. In winter, *pmo*A was only detected in a well defined layer between 6 and 16 cm depth, with the highest PCR product yield between 14 and 16 cm depth (Fig. 3B). No *pmo*A was detected in the upper 6 cm soil depth. In summer, on the other hand, *pmo*A was detected and amplified between 0 cm and 28 cm soil depth, with highest PCR product yield in 6-8 cm depth (Fig. 3B). The *pmo*A gene was only detected in soil layers with CH_4 oxidation activity, indicating that the presence of the *pmo*A gene, and thus methanotrophs, coincided with measured oxidation activity (Fig. 2A & 3B).

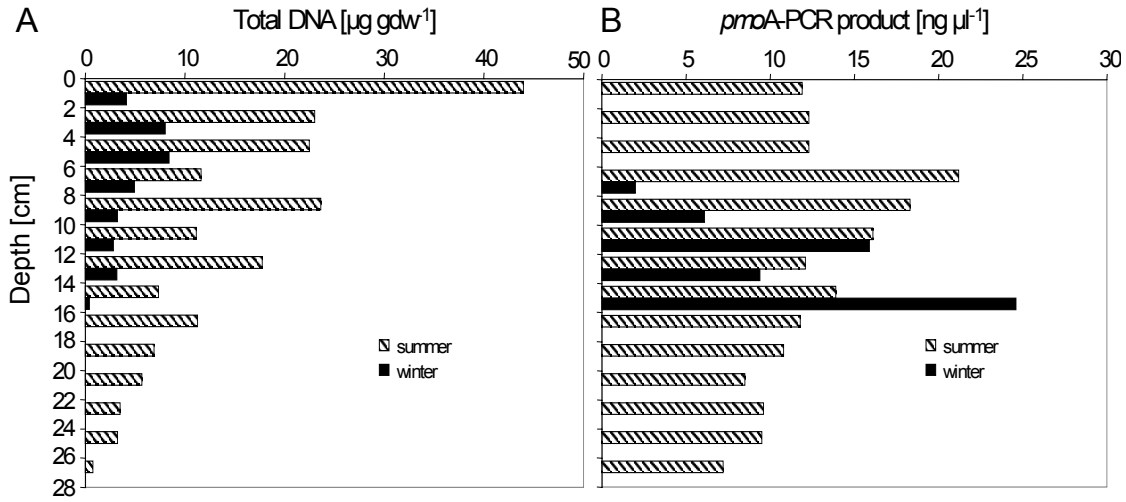

Figure 3. Vertical profiles of (A) the total extractable DNA content per gram dry soil, and (B) the *pmo*A concentration of the PCR products from each soil section (black bars in winter; hatched bars in summer). In winter, DNA was only analyzed between 0 and 16 cm depth; no *pmo*A was detected between 0 and 6 cm depth.

SSU rDNA DGGE community pattern. The DGGE analysis of PCR products amplified with the universal primer set was only conducted in winter (0 - 16 cm depth) and revealed a complex banding pattern, reflecting the high microbial diversity expected for forest soil (Fig. 4A). The number of DGGE bands and the intensity of the bands increased below a soil depth of 2 cm. The DGGE banding pattern changed in different soil depths, indicating changes of the bacterial community structure with depth. The intensity of the DGGE bands indicated about 11 dominant populations within the layer of highest diversity (6 cm to 10 cm depth). Sequence analysis of 5 major DGGE bands revealed two populations (MR(UNI)4, MR(UNI)5) grouping within the phylum of High-GC-gram positive *Bacteria*, two populations (MR(UNI)1, MR(UNI)3) closely related to the phylum *Holophaga-Acidobacterium*, and one population (MR(UNI)2) grouping within the α-*Proteobacteria* (Fig. 4B). The latter population (MR(UNI)2) was closest related to the clones ms14, ms6 and ms10 which were retrieved from a peat bog with primers specific for methanotrophs (Accession numbers: AF111789, AF111787, AF111788)(32).

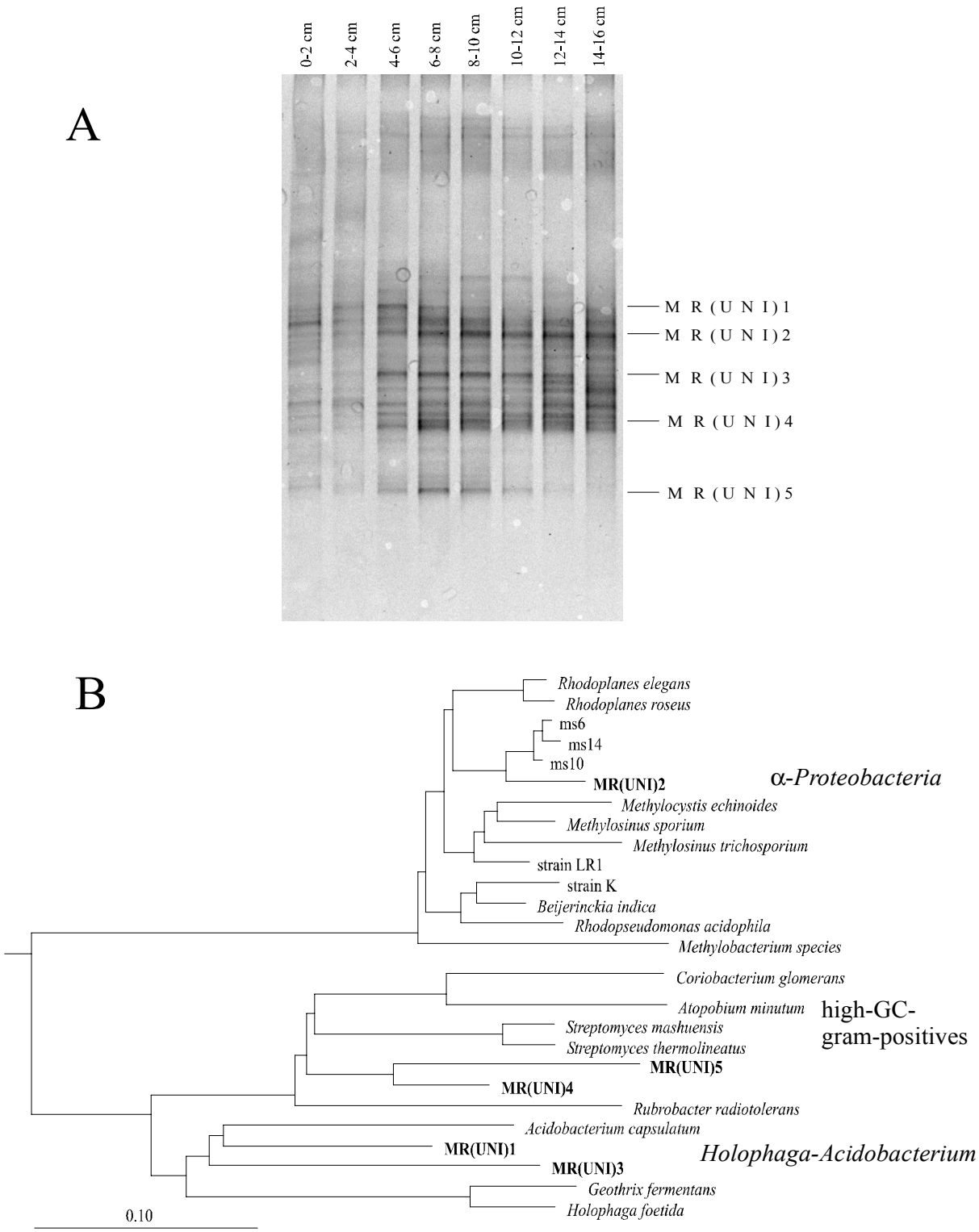

Figure 4. (A) DGGE pattern obtained with the universal SSU rDNA primer set and (B) phylogenetic tree constructed with partial 16S rDNA sequences of the marked DGGE bands from the forest soil core sampled in winter. The phylogenetic tree shows the relationships of the marked DGGE bands to members of the *Bacteria*. The scale bar indicates the estimated number of base changes per nucleotide sequence position; the following 16S rDNA sequences (accession numbers) were taken from data banks: ms6, ms10, ms14 are clones from a peat bog retrieved with primers specific for methanotrophs (AF111789, AF111787, AF111788); strain LR1 is a high-affinity type II methanotroph (Y18442); strain K is an acidophilic methanotroph (Y17144).

***PmoA* DGGE community pattern**. The DGGE analysis of PCR products amplified with the pmoA primer set was performed on soil samples taken in winter (0-16 cm depth) and in summer (0-28 cm depth). In winter, DGGE bands of *pmo*A products were detected between 6 and 16 cm soil depth (Fig. 5A). The number of DGGE bands increased from one to six bands between 6 and 16 cm depth (Fig. 5A). In the soil layer (10-14 cm depth) with highest CH_4 oxidation rates the DGGE bands MR2, MR3, MR4 and MR5 were most intense.

In summer, DGGE bands of *pmo*A were detected between 0 and 28 cm soil depth. The number of DGGE bands increased from one to six bands between 0 and 16 cm depth, then decreased again to only one band below 24 cm depth. The soil layers with maximal CH_4 oxidation activity (4 - 12 cm depth) (Fig. 2A) coincided with those showing the highest PCR product yield of *pmo*A (6 - 12 cm depth) (Fig. 3B) and those with the most intensive DGGE bands (MR2, MR3 and MR5) (6 - 12 cm depth) (Fig 5B). Band MR2 was present in all depth layers, indicating that this was the most dominant and widely distributed methanotrophic population. Band MR4 was most intense in 12 - 24 cm depth (Fig. 5B). Band MR1 appeared only below 14 cm depth, both in winter and summer.

Sequence analysis was performed with the DGGE bands MR1 to MR5 (Fig. 6). DGGE band MR1 grouped among the β-*Proteobacteria* closely related to the ammonium-oxidizing genus *Nitrosospira*. DGGE bands MR2-MR5 formed a distinct group of methanotrophs distantly related to common type II methanotrophs (α-*Proteobacteria*). These sequences were very similar and clustered together with *pmo*A clone sequences (Rold 1, 3, 5; Maine 6, 8 and RA14) recently retrieved from other forest soils (24).

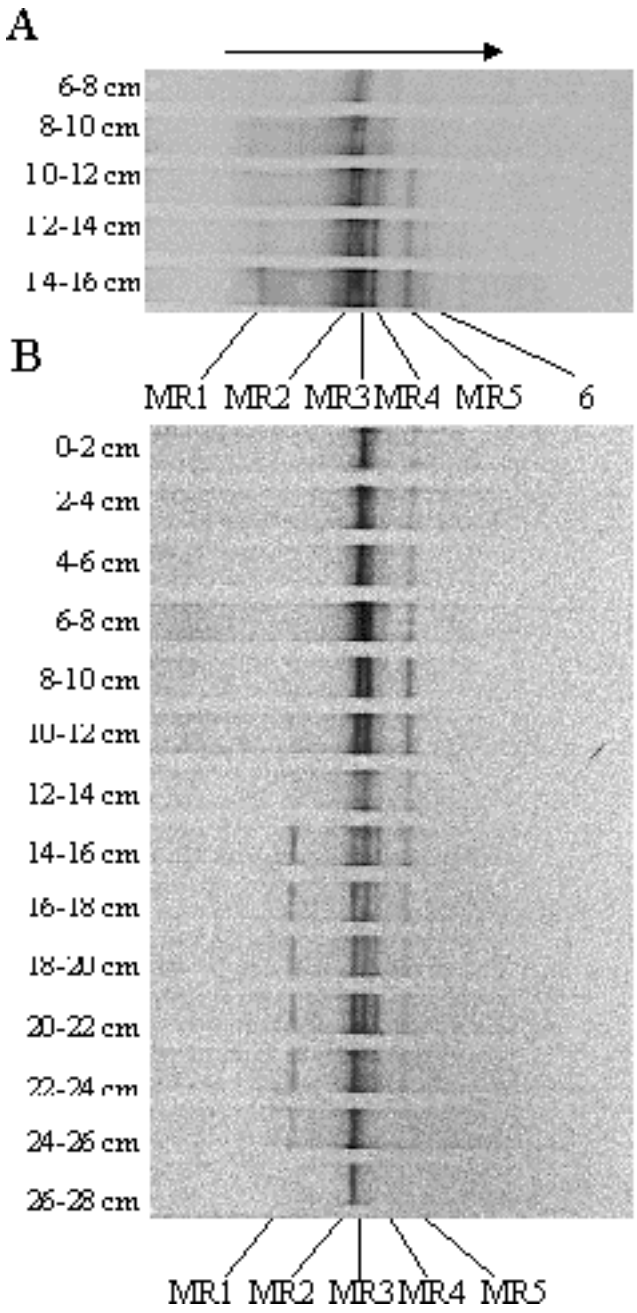

Figure 5. DGGE pattern obtained with the pmoA primer set targeting the gene of the α-subunit of pMMO (A) in winter and (B) in summer. The arrow shows the direction of increasing denaturant and electric field. Bands MR1 to MR5 were sequenced, band 6 was not sequenced

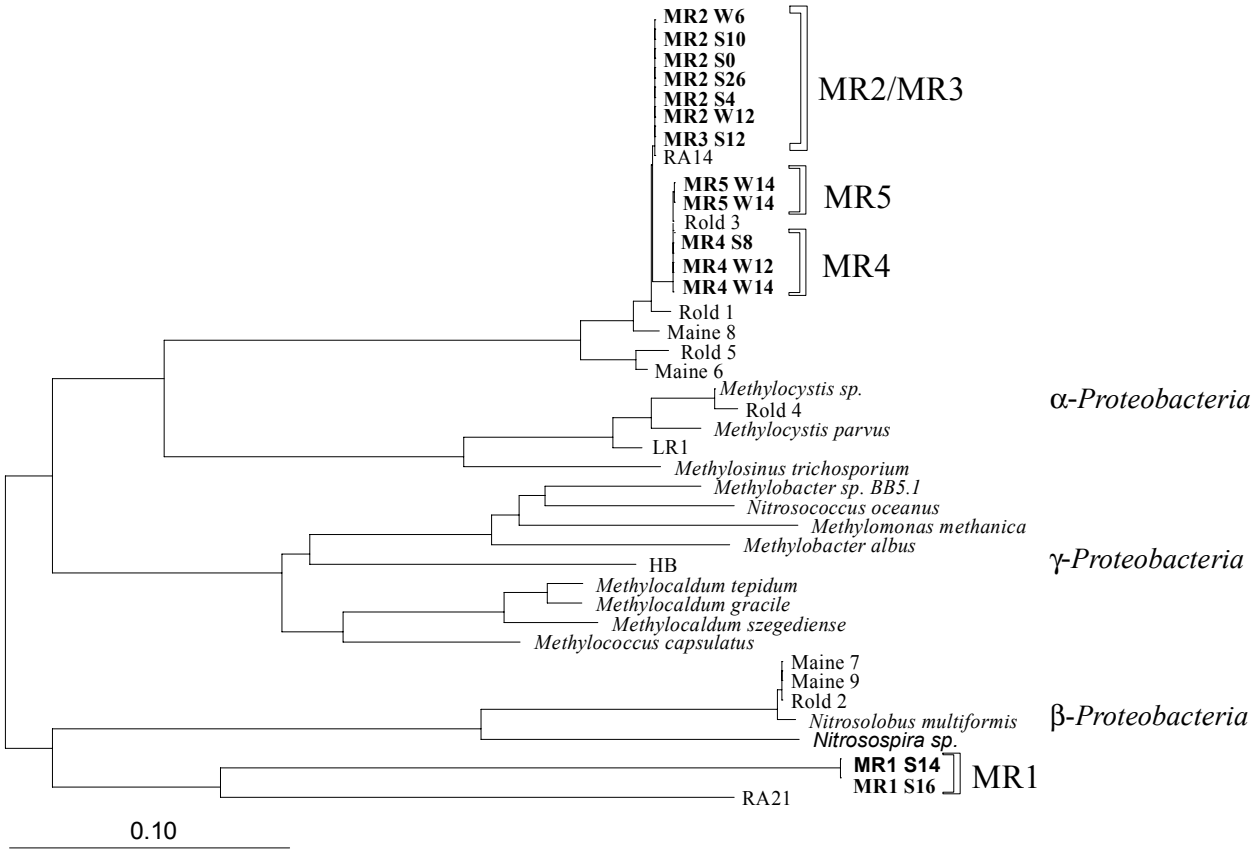

Figure 6. Phylogenetic tree based on the derived amino acid sequences of *pmo*A/*amo*A fragments, showing the relation of the *pmo*A sequences (MR1 to MR5) retrieved from forest soil to *pmo*A and *amo*A sequences of other methanotrophs and ammonium oxidizers. Bands of same electrophoretic mobility were repeatedly sequenced from different DGGE gels and lanes (soil depths) to prove sequence identity. Sequences were labeled according to the DGGE band position (MR1 - MR5), the season of sampling (W, winter; S, summer), and the soil depth (in cm). The scale bar indicates the estimated number of changes per amino acid sequence position. The *pmoA* sequences "Rold", "Maine", and "RA" are novel *pmoA* sequences retrieved from forest soils (24); "LR1" is a high-affinity type II methanotroph (Y18443); "HB" is a thermophilic methanotroph (U89302).

Discussion

Atmospheric CH_4 oxidation has been extensively studied in various upland soils. However, little is known about the methanotrophic community involved. By studying the vertical distribution of both the oxidation of atmospheric CH_4 and the molecular characteristics of methanotrophic populations in an acidic forest soil, we detected a methanotrophic community distinct from known type I and II methanotrophs and only occurring in the soil layers which were actively oxidizing atmospheric CH_4. The *pmo*A sequences of this methanotrophic community clustered distantly away from the known methanotrophs and grouped with *pmo*A clone sequences recently retrieved from other forest soils involved in atmospheric CH_4 consumption (24).

The forest soil examined in this study consumed atmospheric CH_4 at all times. The soil showed a linear decrease of CH_4 with depth, and as reported for other forest soils maximal CH_4 consumption occurred in subsurface soil layers (1, 30, 35, 41). In winter, no CH_4

consumption was measured in the surface layer (0 to 6 cm soil depth) and no methanotrophs were detected by *pmo*A. Here, one reason for the missing CH_4 consumption might be the high the high water content in winter, which exceeded saturation (>100% WHC). Methane was not produced in the soil in contrast to observations in other upland soils (13, 29). Therefore, atmospheric CH_4 rather than endogenously produced CH_4 was the only substrate for CH_4-oxidizing bacteria in this forest soil.

The inhibition of CH_4 oxidation in surface soil was often attributed to high NH_4^+ concentrations (17, 27, 38). The primer set pmoA also amplifies the *amoA* gene of ammonium oxidizers coding for the α-subunit of the ammonium monooxygenase (23). In this forest soil, population MR1, closely related to *Nitrosospira*, was detected below the maximal CH_4 consumption zone, while no ammonium-oxidizers were detected near the surface. The presence of ammonium-oxidizers below the soil layer of maximal CH_4 oxidation activity raised the question whether ammonium-oxidizers were sustained by nitrification. However, the NH_4^+ concentrations were not inhibitory to the methanotrophs. Recently, NH_4^+ was actually identified as a prerequisite for CH_4 oxidation in rice field soil (8).

The increase of total extractable DNA indicated a stimulation and proliferation of all life from January (winter) towards July (summer). However, while the total DNA concentration decreased with depth, the *pmo*A PCR product concentration and the number of methanotrophic populations (i.e. the number of DGGE bands) increased. The methanotrophic community expanded its active zone from a well defined subsoil layer in winter to the entire soil core, in summer. Band MR2 was present in all depth layers, indicating that this was the most widely distibuted methanotrophic population. In the soil layers with highest CH_4 oxidation activity, populations MR2, MR3 and MR5 were prevalent over MR4, whereas MR4 and the putative ammonium-oxidizer MR1 appeared in the soil layers below the maximal oxidation activity. This indicated that the methanotrophic populations were probably adapted to the conditions at a particular depth, each occupying an own ecological niche. The DGGE community pattern itself and the sequences detected were the same in winter and summer.

Dominant bacteria of the forest soil bacterial community belonged to the phyla *Holophaga-Acidobacterium*, High-GC-gram-positive *Bacteria* and *α-Proteobacteria.* The *α-Proteobacteria* DGGE band appeared only in soil layers with CH_4 consumption and was closest related to clone sequences (ms10, ms8, ms14) retrieved with primers specific for methanotrophs from a peat bog (32). However, there is no direct evidence that any of the populations detected by primers targeting the 16S rDNA, were indeed methanotrophs These SSU rDNA sequences, as well as the *pmo*A sequences were distantly related to type II methanotrophs. Recently, phospholipid fatty acid (PLFA) analysis and sequence comparison of *pmo*A in various soils also indicated novel methanotrophs distantly related to type II

methanotrophs and α-*Proteobacteria* (24). Although, the correspondence between SSU rDNA and *pmo*A data might be coincidental, it allows the hypothesis that the *α-Proteobacteria* population, detected with the universal primer set and the methanotrophic populations detected with pmoA are the same. The *α-Proteobacteria* population belonged to the most abundant bacteria in soil layers with CH_4 oxidation. The novel sequences obtained with pmoA PCR-DGGE were found in the same soil layers. Finally, cloning results in other forest soils indicated the same novel *pmoA* sequences to be dominant and ubiquitously distributed in forest soils (24).

Process data and kinetic properties of CH_4 consumption have repeatedly suggested novel methanotrophs to be responsible for atmospheric CH_4 consumption (5, 6, 35). The novel *pmoA* sequences strongly support the existence of yet unknown methanotrophs involved in atmospheric CH_4 consumption as postulated by Bender & Conrad (1992) (6). Radioactive labeling studies showed that high-affinity methanotrophs in forest soils contain unusual i17:0, a17:0 and 17:1ω8c PLFAs, whereas known methanotrophs contain 18:1ω8c and 16:1ω8c PLFAs (36).

Enrichments with high CH_4 concentrations have resulted in the isolation of common methanotrophs from neutral environments. Molecular data from neutral soils and at high CH_4 concentrations demonstrate the presence of common type I and II methanotrophs (20, 25, 34). A novel methanotrophic strain S6, closely related to the genus *Beijerinckia*, was isolated from an acidic blanket peat, also by applying high CH_4 concentrations (12). It is unknown whether this strain, which contains only a soluble methane monooxygenase (sMMO), is able to oxidize CH_4 with high affinity (nanomolar K_m). Enrichments under low CH_4 concentrations resulted in the isolation of the high-affinity type II methanotrophic strain LR1, however, from neutral soil (14).

In summary, all these observations suggest that known type I and II methanotrophs prevail in neutral environments, preferentially at high CH_4 concentrations, but able to adapt to high-affinity kinetics (type II methanotrophs). However, unknown high-affinity methanotrophs seem to prevail in acidic environments oxidizing atmospheric CH_4. The missing link of all the information is the isolation of a bacterium that combines all the molecular evidence, unusual *pmo*A sequence and PLFA pattern, with a high-affinity CH_4 oxidation kinetic. Forest soils tend to be acidic and the unknown, high-affinity methanotrophs seem to thrive at a low pH, and might therefore have escaped isolation in neutral media before (5, 11). This new knowledge about the dominance and the distribution of novel acidicophilic methanotrophs in forest soil, together with the molecular tools at hand, should facilitate enrichment and isolation of the bacterium which constitutes the missing link.

Acknowledgements

The authors thank Bianca Wagner, Sonja Fleissner and Axel Fey for excellent technical assistance. This work was supported by grant BIO-4-CT-960419 from the European Commission.

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V. Zusammenfassende Diskussion

Die vorliegenden Arbeiten geben einen Einblick in die Populationsstruktur, Diversität und Funktion der methanotrophen Lebensgemeinschaft in einem italienischen Reisfeldboden und einem sauren Waldboden. Dazu wurden kultivierungsunabhängige molekularökologische und prozessorientierte biogeochemische Methoden kombiniert, um Funktion und Identität der bakteriellen Lebensgemeinschaft in den untersuchten Habitaten und unter den gegebenen Fragestellungen aufzuklären.

Die mikrobielle Methanoxidation ist entscheidend im globalen Methanhaushalt (siehe Einleitung). Der Prozess der Methanoxidation in Böden ist gut untersucht, doch war über die dafür verantwortliche mikrobielle Lebensgemeinschaft bisher nur wenig bekannt.

Die methanotrophe Lebensgemeinschaft wurde durch fünf PCR-DGGE-Systeme auf Basis dreier Zielregionen innerhalb des Gens der 16S rRNA und zweier funktioneller Gene analysiert. Für die 16S rDNA wurde ein universelles Primerpaar (*Bacteria* und *Archaea*) „Universal“ zur Amplifikation verwendet, sowie zwei spezifischere Primerpaare zur Amplifikation methylotropher Bakterien innerhalb der γ-*Proteobacteria* „MB10γ“ und α-*Proteobacteria* „MB9α“. Zwei weitere Primerpaare amplifizierten die funktionellen Gene *pmo*A und *mxa*F, die für Untereinheiten der pMMO und der MDH kodieren. Die verschiedenen DGGE-Fingerabdrücke in Verbindung mit den Sequenzanalysen der einzelnen Banden lieferten ein detailliertes Bild der vorhandenen methanotrophen Lebensgemeinschaft. Radioaktive Markierungsexperimente und anschließende PLFA-Analysen zeigten, in welchem Ausmaß Typ I und Typ II MOB an der CH_4-Oxidation aktiv beteiligt waren und ermöglichten die Quantifizierung der methanotrophen Biomasse.

1. Die methanotrophe Populationsstruktur im Reisfeldboden

Die im Reisfeldboden bestimmte methanotrophe Lebensgemeinschaft bestand aus Populationen der bekannten MOB des Typs I und II. Die dort gefundenen Typ I MOB waren nahe verwandt zu den Gattungen *Methylobacter* und *Methylococcus* innerhalb der γ-Proteobakterien. Die gefundenen Typ II MOB wurden der *Methylosinus*/*Methylocystis* Gruppe innerhalb der α-Proteobakterien zugeordnet. Methan (>200 ppmv) wird vom Reisfeldboden in einem zweiphasigen Verlauf oxidiert (Bender & Conrad, 1994, 1995; Henckel & Conrad, 1998, Henckel *et al*., 1999, submit; Bodelier *et al*., 2000). Die Oxidation begann mit einer mäßigen Initialoxidationsrate und ging dann in eine zweite, bis zu 40-fach schnellere, induzierte Oxidationsrate über. Der Verlauf der Induktion wird stark vom initialen CH_4 Mischungsverhältnis und von physikochemischen Bedingungen des Bodens beeinflusst (Bender & Conrad, 1995). Die Induktion der CH_4-Oxidation kann entweder durch eine Akti-

vitätsänderung der MOB, durch eine Populationsvergrößerung oder einer Kombination aus beidem erfolgen. Bei CH_4 Mischungsverhältnissen >7000 ppmv konnte ein Wachstum von MOB durch MPN Zählungen festgestellt werden (Bender & Conrad, 1994; 1995).

Der starke Konzentrationsanstieg an für MOB spezifischen PCR-Produkten und eine ausgeprägte Intensitätssteigerung der MOB spezifischen DGGE-Banden in induziertem Reisfeldboden wurde auf eine Populationsvergrößerung zurückgeführt (Henckel *et al*., 1999). Der bis zu 30-fache Anstieg der Typ I und Typ II spezifischen PLFAs verifizierte diese Annahme (Henckel *et al*., submit; Bodelier *et al*., 2000). Die Induktion der CH_4-Oxidation war auch bei geringen CH_4 Mischungsverhältnissen mit einer deutlichen Zunahme an methanotropher Biomasse verbunden.

1.1. Effekt von O_2 und CH_4

In wassergesättigten Böden wird die CH_4-Oxidation unmittelbar durch die Verfügbarkeit von O_2 und CH_4 reguliert (Schimel *et al*., 1993). Wie zu erwarten, wurde die methanotrophe Lebensgemeinschaft im Reisfeldboden durch CH_4 und O_2 stimuliert (Henckel *et al*., 1999). Die Verfügbarkeit von O_2 und CH_4 hat vermutlich auch einen Einfluss auf die Zusammensetzung und Aktivität der methanotrophen Lebensgemeinschaft.

Um diesen Einfluss zu untersuchen, wurde Reisfeldboden in Durchflusssystemen unter vier unterschiedlichen CH_4 /O_2 Atmosphären kontinuierlich inkubiert. Die künstlichen Atmosphären bestanden aus den Kombinationen von niedrigem (1000 ppmv) oder hohem (10000 ppmv) CH_4 mit jeweils niedrigem (1 %) und hohem (atmosphärisch ≅ 20,5 %) O_2 Mischungsverhältnis.

Anhand des CH_4-Oxidationsverlaufes und der PLFA-Analysen zeigte sich, dass atmosphärischer O_2 (20,5 %) inhibierend auf Wachstum und Aktivität der methanotrophen Lebensgemeinschaft besonders im Zusammenhang mit niedrigem CH_4 wirkte. Wohingegen niedriger O_2 (1 %) weder Aktivität noch Biomassezuwachs der methanotrophen Lebensgemeinschaft einschränkte. Sauerstoffempfindlichkeit war schon vorher an Reinkulturen von MOB und an CH_4-Oxidationsraten in Seen beobachtet worden (deBont & Mulder, 1974; Whittenbury *et al*., 1975; Rudd & Hamilton, 1975). MOB wurden daher als fakultativ mikroaerophil beschrieben (Rudd & Taylor, 1980). In Seen wurde die O_2-Inhibiton durch N-Eintrag aufgehoben, was zu der Vermutung führte, dass nicht die CH_4-Oxidation, sondern die N_2-Fixierung der O_2 sensitive Prozess ist (Rudd *et al*., 1976). Wir verwendeten jedoch AMS-Medium zur Befeuchtung des Bodens, um eine N-Limitation zu vermeiden. Auch das Vorkommen aktiver Typ I Populationen, die nicht N_2 fixieren können, ließ eine N-Limitation im Boden als Grund für die O_2-Inhibition sehr unwahrscheinlich erscheinen (Henckel *et al.*, submit).

Auf die Populationsstruktur von Typ II MOB hatte die unterschiedliche Verfügbarkeit von CH_4 und O_2 nur geringen Einfluss. Die mit MB9α bestimmte Populationsstruktur von Typ II MOB veränderte sich nicht. Nach mehreren Tagen unter hohem CH_4 wurden, unabhängig vom O_2-Gehalt, Typ II Populationen aktiv und jeweils eine neue Typ II Population mit den Primerpaaren „Universal“ und „pmoA“ detektiert.

Ganz im Gegensatz zur fast unveränderten Populationsstruktur der Typ II MOB, änderte sich die Populationsstruktur der Typ I MOB unter den verschiedenen O_2/CH_4-Atmosphären sehr stark und mit einer hohen Dynamik. Unter niedrigem CH_4 zeigte das MB10γ DGGE-Bandenmuster für Typ I MOB eine höhere Diversität der Populationsstruktur als unter hohem CH_4. Bestimmte Typ I Populationen wurden nur unter hohem CH_4 und niedrigem O_2 durch das funktionelle Primerpaar „mxaF“ (für die MDH) detektiert.

Sowohl unterschiedliche CH_4 als auch O_2 Mischungsverhältnisse hatten demnach einen Einfluss auf Diversität und Populationsstruktur bei Typ I MOB. Bei Typ II MOB wurde hingegen ausschließlich durch hohe CH_4 Mischungsverhältnisse eine leichte Änderung der Populationsstruktur beobachtet, während O_2 keinen sichtbaren Einfluss hatte. Die Zunahme einzelner Banden von Typ II MOB in der DGGE deuteten daraufhin, dass einzelne Populationen durch hohe CH_4 Mischungsverhältnisse im Wachstum stimuliert wurden.

Während die DGGE-Analysen komplexe Änderungen einzelner Typ I und Typ II MOB Populationen in Abhängigkeit von CH_4 oder O_2 Verfügbarkeit zeigten, war der Einbau von $^{14}CH_4$ in PLFAs, also die relative Aktivität der MOB nur durch CH_4 bestimmt. Die Analyse der ^{14}C-PLFAs ergab eine klare Aktivitätsverteilung zwischen den beiden methanotrophen Typen. Über 90 % der Radioaktivität während der Initialoxidation und Induktion wurde in Typ I MOB spezifische PLFAs eingebaut. Typ II MOB wurden nur unter hohen CH_4 und sehr viel später als Typ I MOB aktiv. Die Gesamtbiomasse (Typ I + Typ II) war unter hohen CH_4 Mischungsverhältnissen erwartungsgemäß höher als unter niedrigen CH_4 Mischungsverhältnissen, wobei der Anteil der Typ I Biomasse unter niedrigen CH_4 höher war als der Typ II Biomasse.

1.2. Vertikale Verteilung der MOB nach Drainage

In gefluteten Reisfeldern findet CH_4-Oxidation nur an oxisch-anoxischen Grenzflächen in der oberen 2-mm Bodenschicht und in der Rhizosphäre statt (Conrad & Rothfuss, 1991; Frenzel *et al*., 1992; King, 1996; van der Gon *et al*., 1996; Bosse & Frenzel, 1997, 1998). Bei der Drainage dringt mit sinkendem Wassergehalt O_2 in den Boden ein, wodurch die Methanproduktion stark abnimmt (Sigren *et al*., 1997; Ratering & Conrad, 1998). Die veränderten CH_4 /O_2 Konzentrationsgradienten im Bodenprofil lassen eine Auswirkung auf die Verteilung der methanotrophen Lebensgemeinschaft in verschiedenen Bodentiefen erwarten.

Die Populationsstruktur der Typ II MOB in den unterschiedlichen Bodentiefen änderte sich geringfügig mit der Drainagedauer. Bereits einen Tag nach Drainage waren in den noch wassergesättigten und anoxischen Tiefenschichten Typ II MOB vorhanden, die offensichtlich unter diesen Bedingungen überleben können. Acht Tage nach Beginn der Drainage wurden zwei zuvor noch nicht detektierte Typ II Populationen gefunden. Das war ein Indiz dafür, dass einige Typ II MOB durch Drainage aktiviert und im Wachstum stimuliert wurden.

Die Zusammensetzung der Populationsstruktur von Typ I MOB veränderte sich während der Drainage sehr viel stärker als die der Typ II MOB. Einen Tag nach Drainage waren im noch wassergesättigten Boden Typ I MOB nicht oder nur schwach in den obersten 4 mm Boden detektierbar. Während der Drainage kam es dann zu einer starken Stimulation und Wachstum von Typ I Populationen. Nach 8 Tagen waren in allen Bodenschichten bis 20 mm Tiefe Typ I MOB dominant vorhanden. Die Diversität der Typ I Populationsstruktur veränderte sich in fast jeder der getesteten 2-mm Bodensegmente. Zu dieser Zeit sollte sich ein gegenläufiger CH_4 -O_2 -Gradient im Boden ausgebildet haben, in welchem sich die einzelnen Typ I Populationen etablierten.

Dieses Ergebnis und die Ergebnisse aus den Inkubationen unter unterschiedlichen O_2 / CH_4 Mischungsverhältnissen lassen vermuten, dass die einzelnen Typ I Populationen jeweils enge Wachstumsoptima besitzen, wodurch sich jeweils differenzierte Populationsstrukturen unter gegebene Bedingungen ausbilden.

1.3. Effekt von Düngung und Rhizosphäre auf die MOB

Neben CH_4 und O_2 hat NH_4^+ großen Einfluss auf die CH_4-Oxidation. In vielen Böden und Sedimenten wurde eine Unterdrückung oder Hemmung der CH_4-Oxidation auf NH_4^+ zurückgeführt (Steudler *et al*., 1989, King & Schnell, 1994; Gulledge *et al*. 1997; Bosse *et al*., 1993; van der Nat *et al*., 1997; Hütsch *et al*., 1996, Hütsch, 1998). In der Reisrhizosphäre hatte NH_4^+ jedoch einen stimulierenden Effekt auf die methanotrophe Lebensgemeinschaft und führte dadurch zu einer höheren CH_4-Oxidationsrate und zu einer verminderten CH_4 -Freisetzung in die Atmosphäre (Bodelier *et al*., 2000).

DGGE-Analysen in bepflanzten Reismikrokosmen ergaben, dass in der Rhizosphäre sowie im anoxischen, nicht-durchwurzelten Boden eine stabile unveränderliche Typ II Lebensgemeinschaft vorhanden war. Die Populationsstruktur der Typ II MOB veränderte sich auch nicht durch Düngungen mit NH_4^+ oder Harnstoff in unterschiedlichen Konzentrationen.

Die Typ I MOB hingegen waren überwiegend in der Rhizosphäre vorhanden und nicht, oder nur sehr schwach, im nicht-durchwurzelten, anoxischen Boden. Dies kann, wie auch bei den Untersuchungen mit unterschiedlichen O_2 / CH_4 Mischungsverhältnissen und

bei Drainage von Reisfeldboden gezeigt, auf die O_2 Verfügbarkeit in der Rhizosphäre zurückgeführt werden. Typ I MOB waren nur dort vorhanden wo CH_4-Oxidation durch O_2 ermöglicht wurde. Die verschiedenen N-Düngungen bewirkten Veränderungen der Typ I Populationsstruktur gegenüber ungedüngten Kontrollen, die aber nicht eindeutig einer Stickstoffart (NH_4^+ oder Harnstoff) zugeordnet werden konnten.

Typ II MOB waren numerisch (MPN-Zählungen) abundanter und produzierten bis zu 7-fach mehr PLFA Biomasse im Reismikrokosmos als Typ I MOB (Bodelier *et al*., 2000). Typ I MOB wurden jedoch durch die N-Düngung relativ stärker im Wachstum stimuliert als Typ II MOB.

Methanotrophe des Typ II sowie die Typ I MOB *Methylococcus sp*. und *Methylomonas sp*. können N_2-fixieren, während dem Großteil der Typ I MOB diese Möglichkeit fehlt. Es wurde daher postuliert das Stickstoffverfügbarkeit zugunsten von Typ I MOB selektiert (Hanson & Hanson, 1996). Der relativ höhere Biomassezuwachs der Typ I MOB durch N-Düngung scheint dies im Reisfeldboden zu bestätigen (Bodelier *et al*., 2000).

1.4. Abundanz der MOB im Reisfeldboden

Typ II MOB sind ungleich häufiger aus verschiedenen Habitaten angereichert oder isoliert worden als Typ I MOB, was vermuten lässt, dass Typ II MOB allgemein abundanter als Typ I MOB in der Umwelt vorkommen (Hanson & Hanson, 1996).

Im Reisfeldboden konnte diese Vermutung bestätigt werden. In den höchsten positiven MPN Verdünnungsstufen aus Reismikrokosmen wurden nur Typ II MOB detektiert (Bodelier *et al*., 2000). Auch die PLFA-Analyse zeigte, dass Typ II spezifische 18:1ω6 PLFA in bis zu 7-fach höherer Konzentration vorkamen als Typ I spezifische 16:1ω6 PLFAs (Bodelier *et al*., 2000; Henckel *et al*., submit). Nur wenn der Reisfeldboden länger unter niedrigem CH_4 Mischungsverhältnis inkubiert wurde, überwog die Typ I PLFA Biomasse und Typ I MOB waren abundanter (Henckel *et al*., submit).

Im Reisfeldboden konnten über MB9α und mxaF DGGE-Analysen mehrfach die gleichen Typ II Populationen detektiert werden. Auch die aus Reisfeldboden isolierten Typ II Stämme RP1 und W5 wurden mit mxaF detektiert (Gilbert *et al*., 1998). Dies deutet daraufhin, dass diese Organismen gut überlebensfähig sind und dominante Vertreter der methanotrophen Lebensgemeinschaft in italienischen Reisfeldern darstellen.

Methanotrophe Bakterien bilden Exosporen oder Cysten als Überdauerungsformen (Whittenbury, 1970). Während Typ II MOB hitzeresistente und mehrere Monate überdauerungsfähige Exosporen (*Methylosinus*) oder Lipid-Cysten (*Methylocystis*) bilden, besitzen Typ I MOB nur zum Teil „Azotobacter-ähnliche“ Cysten (*Methylomonas, Methylococcus)*, die nur einige Wochen überdauerungsfähig und nicht hitzeresistent sind (Whittenbury *et al*., 1970; Reed & Dugan, 1979; Reed, *et al*., 1980; Titus *et al*., 1982). Typ II MOB schei-

nen allgemein besser zu überleben und in Habitaten zu dominieren in den Wachstum periodisch durch ungünstige Umweltbedingungen unterbrochen ist, während Typ I MOB in Habitaten überwiegen, die schnelles Wachstum ermöglichen (Vecherskaya *et al*., 1993; Hanson & Hanson, 1996, Roslev & King, 1994).
Reisfelder liegen einige Monate im Jahr trocken, sind jedoch im gefluteten Zustand durch hohe CH_4-Produktion und relativ homogene Bedingungen charakterisiert. Damit bieten sie an den oxisch-anoxischen Grenzflächen einen optimalen Lebensraum für Typ II MOB, die längere ungünstige Umweltbedingungen überdauern können. In kurzen Phasen während der Bewirtschaftung der Felder, z. B. bei der zwischenzeitlichen oder endgültigen Drainage vor der Ernte, nimmt die CH_4-Produktion ab und die Bodenstruktur wird heterogener. Unter solchen Bedingungen, sowie in der Rhizosphäre bei ausreichender NH_4^+-Verfügbarkeit, werden Typ I MOB gefördert.

2. Die methanotrophe Populationsstruktur im Waldboden

Die Oxidation von atmosphärischem CH_4 in oxischen Böden ist die einzige terrestrische Senke für CH_4 und übernimmt daher eine wichtige Funktion im globalen CH_4-Haushalt. Die kinetischen Parameter der CH_4-Oxidation in oxischen Böden lassen auf die Existenz noch unbekannter hoch-affiner Methanoxidierer schließen (Bender & Conrad, 1992; Conrad, 1996; 1995 a). Das atmosphärische CH_4-Oxidationsmaximum liegt im Waldboden typischerweise unterhalb der Oberfläche in ca. 10-15 cm Tiefe, im oberen Mineralboden (Whalen *et al*., 1992; Koschorreck & Conrad, 1993; Adamsen & King, 1993; Roslev *et al*., 1997).

Der hier untersuchte Waldboden war eine saure Braunerde (≈ pH 4) und oxidierte atmosphärisches CH_4 im Winter zwischen 6 und 16 cm Bodentiefe und im Sommer in einer Tiefe von 0-26 cm. Das Oxidationsmaximum lag auch hier unterhalb der Oberfläche zwischen 10 und 14 cm im Winter und 2 bis 6 cm im Sommer. Das Gen für die pMMO (*pmo*A) wurde nur in Bodenschichten detektiert, in denen atmosphärisches CH_4 oxidiert wurde. Im DGGE-Bandenmuster zeigte sich, dass die Anzahl der *pmo*A-Banden in den Bodenschichten mit maximalen CH_4-Oxidationsraten höher war als in höher oder tiefergelegenen Bodenschichten, in denen CH_4 mit geringeren Raten oxidiert wurde. Viele MOB besitzen zwei pMMO Kopien im Genom, dadurch kann die Anzahl der detektierten *pmo*A-Banden höher sein als die tatsächliche Anzahl detektierter Populationen (Semrau *et al*., 1995). Dennoch korrelierten die höheren Oxidationsraten mit einer größeren Anzahl an MOB Populationen. Die *pmo*A-Sequenzen waren nur entfernt verwandt mit den *pmo*A-Sequenzen bekannter Typ II MOB und stellen wahrscheinlich eine neue, unbekannte Gruppe acidophiler methanotropher Populationen dar. Sehr nahe verwandte *pmo*A-Sequenzen wurden in anderen Waldböden gefunden (Holmes *et al*., 1999; Roslev *et al*., 1999). Radioaktive Markierungs-

experimente und PLFA-Analyse ergaben, dass die unbekannten, hoch-affinen Methanoxidierer in den Waldböden auch 17:0 und 17:1ω8c PLFAs enthalten, die bei den bereits bekannten MOB nicht vorkommen (Roslev *et al*., 1999).

2.1. Hoch-affine Methanotrophe

Obwohl in sauren Waldböden unbekannte hoch-affine MOB detektiert wurden, ist hoch-affine Methanoxidation nicht, wie ursprünglich postuliert, auf unbekannte Methanoxidierer beschränkt. Der aus einem oxischen Humisol isolierte Stamm LR1 ist in der Lage niedrige CH_4 Mischungsverhältnisse im nanomolaren Bereich zu oxidieren (Dunfield *et al*., 1999). Je nach Wachstumsbedingungen wechselt LR1 zwischen einem apparenten mikropolaren oder nanomolaren K_m Wert. Wie durch Sequenzierung der 16S rDNA, und der funktionellen Gene *pmo*A und *mxa*F gezeigt wurde, ist LR1 nahe verwandt mit der Typ II MOB Gattung *Methylocystis* (Dunfield *et al*., 1999).

Es ist nicht auszuschließen, dass auch andere bereits bekannte MOB in der Lage sind, ihr Enzymsystem auf sehr niedrige, im nanomolaren Bereich liegende CH_4 Konzentrationen adaptieren zu können.

3. Abschließende Betrachtung

Methanotrophe sind ubiquitär verbreitet und wurden in Böden, Sedimenten und Mauerwerk, genauso wie in marinen und aquatischen Lebensräumen fast aller Klimazonen gefunden (Heyer *et al*., 1984; Hanson & Hanson, 1996). Relativ häufiger wurden Typ II MOB, sowohl durch kultivierungsabhängige als auch unabhängige Methoden nachgewiesen (Hanson & Hanson, 1996; Gilbert *et al*., 1998; McDonald & Murrell, 1997a+b; Overas *et al*.,. 1998; Jensen *et al*., 1998). Auch in dem hier untersuchtem Reisfeldboden waren Typ II MOB abundanter als Typ I MOB. Die Populationsstruktur von Typ II MOB veränderte sich kaum, auch unter der CH_4-Oxidation abträglichen Umweltbedingungen. Die hohen Zellzahlen und die unveränderliche Populationsstruktur unterstützen die Vermutungen, dass Typ II MOB besser überdauerungsfähig und an Habitate angepasst sind, in denen periodisch die CH_4-Oxidation unterbrochen ist.

Typ I MOB hingegen unterlagen großen Populationsschwankungen und waren durch sehr unterschiedliche Zusammensetzung der Populationsstruktur charakterisiert. Unter ungünstigen, z.B. anoxischen Bedingungen waren Typ I MOB nicht oder kaum detektierbar, konnten aber innerhalb weniger Tage eine diverse, je nach Umweltbedingungen unterschiedliche Populationsstruktur ausbilden und über 90 % der CH_4-Oxidationsaktivität abdecken. Typ I MOB erscheinen als Spezialisten, die an ein enges Wachstumsoptimum angepasst sind.

Die beiden methanotrophen Typen scheinen unterschiedliche ökologische Strategien zu verfolgen: Typ II MOB eine Überdauerungsstrategie und als Generalist breit angelegte Wachstumsbedingungen, Typ I MOB spezialisierte Anpassung an bestimmte Wachstumsbedingungen, mit der Fähigkeit unter diesen optimalen Bedingungen sehr schnell aktiv zu werden.

Die bisherige phylogenetische Verbreitung der Methanotrophie in zwei eng vergesellschaftete Gruppen (Cluster) innerhalb der α- und γ-Proteobakterien beginnt sich aufzulösen bzw. auszudehnen. Die gerade neu beschriebene Gattung *Methylocella palustris* ist sowohl phylogenetisch als auch morphologisch von Typ I und II MOB verschieden. *Methylocella palustris* ist acidophil, besitzt nur die sMMO und keine pMMO sowie keine internen Membranstapel. Phylogenetisch ist es nahe verwandt zu den heterotrophen α-Proteobakterien *Bejerinckia* und *Rhodopseudmonas*, mit denen es auch morphologische Merkmale teilt (Dedysh *et al*., 2000).

Die methanotrophen Populationen in Waldböden wurden bisher nur über Klonierung und DGGE-Analyse des *pmo*A-Gens, sowie über PLFA-Markierungsexperimente nachgewiesen (Holmes *et al*., 1999; Roslev *et al*., 1999; Henckel *et al*., in press). Die phylogenetische Plazierung auf Basis der 16S rDNA und die morphologische Beschreibung dieser noch nicht isolierten hoch-affinen und wahrscheinlich auch acidophilen MOB aus Waldböden steht jedoch noch aus. Es gilt abzuwarten, ob diese unbekannten Methanoxidierer die momentane Verteilung der Methanotrophie im phylogenetischen System weiter auffächern, wie es die Berechnungen auf Grundlage der *pmo*A vermuten lassen (Henckel *et al*., in press; Holmes *et al*., 1999).

Methanotrophe haben eine sehr ungewöhnliche Enzymausstattung. Typ II und einige Typ I MOB besitzen mit der pMMO und sMMO zwei völlig verschiedene Enzymsysteme für die gleiche Reaktion. Zudem enthalten viele MOB mindestens zwei Kopien der pMMO, die sich in der Aminosäuresequenz leicht unterscheiden (Semrau *et al*., 1995; Stolyar *et al*., 1999). Das Operon der pMMO besteht aus den 3 Genen pmoC, pmoA und pmoB. *Methylococcus capsulatus* (Bath) besitzt je zwei Kopien von pmoA und B, aber drei von pmoC (Stolyar *et al*., 1999). „Knockout" Mutanten der Genkopie 1 zeigten noch 2/3, der Genkopie 2 lediglich 1/3 der Wildtypaktivität und $pmoC_3$ Mutationen waren letal (Stolyar *et al*., 1999). Die wahrscheinlich unterschiedlichen pMMO Kopien unterscheiden sich auch in ihrer O_2 Empfindlichkeit. Bei Reinigung der pMMO wurden hinsichtlich der O_2 Empfindlichkeit drei verschiedene Varianten unterschieden (Nguyen *et al*., 1998).

Vor dem Hintergrund dieses sehr vielschichtigen und variablen Enzymapparates erscheint es kaum verwunderlich, dass MOB sich den Bedingungen ihres Lebensraumes sehr genau und unterschiedlich anpassen können.

So oxidiert die methanotrophe Lebensgemeinschaft im Reisfeldboden in der Regel hohe Methankonzentrationen und die Oxidation von atmosphärischem CH_4 wird nicht längerfristig aufrecht erhalten. Doch auch in Reisfeldboden konnte eine hoch-affine Kinetik induziert werden (Bender & Conrad, 1994). Hingegen ist der Typ II Stamm LR1, der aus einem Boden isoliert wurde, der überwiegend atmosphärisches Methan aufnimmt aber zwischenzeitlich CH_4 produziert, in der Lage, seinen K_m je nach Wachstumsbedingungen anzupassen und sowohl niedrige als auch hohe CH_4 Mischungsverhältnisse zu oxidieren (Dunfield *et al*., 1999).

Im weiteren Verlauf der CH_4-Oxidation sind Enzyme am C1-Transfer beteiligt, die ursprünglich nur bei methanogenen und sulfatreduzierenden Archaea gefunden wurden (Christoserdova *et al*., 1998; Vorholt *et al*., 1999). Der außergewöhnliche und sehr unterschiedlich ausgeprägte Enzymapparat der MOB kann von einem gemeinsamen Vorgänger stammen, oder durch horizontalen Gentransfer erlangt worden sein (Christoserdova *et al*., 1998). Die unmittelbare räumliche Nähe methanogener Archaea und methanotropher Bakterien in vielen Habitaten und ein damit verbundener möglicher erfolgter horizontaler Gentransfer ist eine reizvolle Hypothese, besonders vor dem Hintergrund der gerade stattfindenden Diskussion über eine vernetzte Phylogenie (Doolittle, 1999; Philippe & Forterre, 1999; Nelson *et al*., 1999).

Die Phylogenie setzt einer Lebenszyklusstrategie und dem Habitat eines Organismus Grenzen (Begon *et al*., 1996). Die Methanotrophie scheint sich diesem zu entziehen. Die phylogenetische Entfernung und die unterschiedlich ausgeprägten Lebensräume und Strategien der MOB lassen einen horizontalen Gentransfer im Zusammenhang mit der Fähigkeit zur Methanoxidation plausibel erscheinen. So könnte man sich vorstellen, dass unterschiedliche Bakteriengruppen zu einem oder mehreren Zeitpunkten in verschiedenen Habitaten (neutral, sauer, hohe CH_4-Konzentration, atmosphärisches CH_4) Eigenschaften zur Methanoxidation übernommen und weiterentwickelt haben. Eine erfolgte laterale Genentwicklung der MOB würde die vielseitige Morphologie, Enzymatik und Ökologie verständlicher erscheinen lassen.

In den vergangenen Jahren ist das Wissen und Verständnis über die Methanoxidation, ihre Phänomene und Herausforderungen, immens angewachsen. Neue molekularbiologische Methoden ermöglichten, kultivierungsunabhängig die Untersuchung der autochthonen Lebensgemeinschaften im Habitat. Dennoch müssen durch, die z. T. weit fortgeschrittene, Isolierung und Anreicherung hoch-affiner und acidophiler MOB, die noch fehlenden Isolate zu den durch die molekularökologischen Untersuchungen aufgezeigten aber nicht hinreichend zu füllenden Lücken geschlossen werden.

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VII. Anhang

1. Hoch-affine Methanoxidation einer Anreicherungskultur aus Boden durch ein methanotrophes Bakterium des Typ II

Hier wird die Anreicherung und Identifikation, eines methanotrophen Bakteriums - Stamm LR1- beschrieben. Die Anreicherung und Identifikation wurde durch PCR-DGGE-Analysen der Gene *pmo*A und *mxa*F unterstützt. Phylogenetische Berechnungen aufgrund der Sequenzen des Gens der 16S rRNA, der *pmo*A und der *mxa*F platzierten LR1 in die Gruppe der Typ II MOB. Der Stamm LR1 ist in der Lage unter sehr niedrigen (<275 ppmv) CH_4 Mischungsverhältnissen zu wachsen. Reinkulturen der bisher bekannten Typ I und Typ II MOB waren nicht in der Lage längere Zeit unter diesen niedrigen CH_4-Mischungsverhältnissen zu überleben.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1999, p. 1009–1014 Vol. 65, No. 3
0099-2240/99/$04.00+0

High-Affinity Methane Oxidation by a Soil Enrichment Culture Containing a Type II Methanotroph

PETER F. DUNFIELD,[1,2] WERNER LIESACK,[1] THILO HENCKEL,[1] ROGER KNOWLES,[2] AND RALF CONRAD[1]*

Max-Planck-Institut für terrestrische Mikrobiologie, 35043 Marburg, Germany,[1] and Department of Natural Resource Sciences, Macdonald Campus of McGill University, Ste. Anne de Bellevue, Quebec, Canada[2]

Received 30 July 1998/Accepted 3 December 1998

Methanotrophic bacteria in an organic soil were enriched on gaseous mixing ratios of <275 parts per million of volume (ppmv) of methane (CH_4). After 4 years of growth and periodic dilution (>10^{20} times the initial soil inoculum), a mixed culture was obtained which displayed an apparent half-saturation constant [$K_{m(app)}$] for CH_4 of 56 to 186 nM (40 to 132 ppmv). This value was the same as that measured in the soil itself and about 1 order of magnitude lower than reported values for pure cultures of methane oxidizers. However, the $K_{m(app)}$ increased when the culture was transferred to higher mixing ratios of CH_4 (1,000 ppmv, or 1%). Denaturing gradient gel electrophoresis of the enrichment grown on <275 ppmv of CH_4 revealed a single gene product of *pmoA*, which codes for a subunit of particulate methane monooxygenase. This suggested that only one methanotroph species was present. This organism was isolated from a sample of the enrichment culture grown on 1% CH_4 and phylogenetically positioned based on its 16S rRNA, *pmoA*, and *mxaF* gene sequences as a type II strain of the *Methylocystis*/*Methylosinus* group. A coculture of this strain with a *Variovorax* sp., when grown on <275 ppmv of CH_4, had a $K_{m(app)}$ (129 to 188 nM) similar to that of the initial enrichment culture. The data suggest that the affinity of methanotrophic bacteria for CH_4 varies with growth conditions and that the oxidation of atmospheric CH_4 observed in this soil is carried out by type II methanotrophic bacteria which are similar to characterized species.

Methane-oxidizing bacteria inhabit the aerobic interfaces of methanogenic environments and reduce the potential methane (CH_4) emissions from these environments (7, 15, 30). Atmospheric CH_4, which has a present mixing ratio of 1.7 parts per million of volume (ppmv), is also oxidized microbially in aerobic upland soils (15). This process represents about 10% of the atmospheric CH_4 sink (10).

The identity of these atmospheric-CH_4 oxidizers is unknown. Whereas soil CH_4 oxidation rates can remain steady for >4 months at 1.7 ppmv of CH_4 (34), calculations based on the kinetic constants of known methanotrophic species suggest that these organisms are incapable of such extended survival (6). Atmospheric CH_4 should not supply sufficient cellular maintenance energy plus reducing power for the methane monooxygenase (MMO) enzyme. Studies with *Methylosinus trichosporium* and *Methylobacter albus* (*Methylomicrobium album*) seem to confirm this (32, 34).

However, the kinetic properties of CH_4 oxidation in upland soils are different from those in pure methanotroph cultures. Apparent half-saturation constants [$K_{m(app)}$] of various type I and II methanotrophs range from 0.8 to 66 μM CH_4 (5, 18). While some of these values are overestimated due to diffusion limitation, a lower limit of 0.8 to 2 μM is probable (18). Environmental samples from the aerobic interfaces of methanogenic habitats, such as lake sediments and landfill cover soils, have $K_{m(app)}$ values in the same range (7, 39). However, the $K_{m(app)}$ values for upland forest and agricultural soils are 1 to 3 orders of magnitude lower, at 10 to 280 nM CH_4 (1, 3, 7, 8, 13). Although a lower-affinity activity can be induced by enrichment with atmospheres containing 10% CH_4 (1), the methanotrophs normally active in these soils seem to be adapted to reduced CH_4 levels. Either uncharacterized species are involved in atmospheric-CH_4 oxidation or unknown physiological changes are induced in known methanotrophic species living in these soils.

The $K_{m(app)}$ for CH_4 consumption in an organic soil from Ottawa, Canada, was estimated as 80 to 90 nM (8). This is in the same order of magnitude as values measured in other aerobic upland soils, although slightly higher. Values as low as 10 nM have been measured in soils (1, 3, 7, 13). Here we report on experiments aimed at enriching and characterizing the organisms responsible for the high-affinity activity in this organic soil.

MATERIALS AND METHODS

Sampling site. The study site has been described previously (8, 9). It is an organic (60% combustible matter), neutral (pH 6.7 to 7.2) soil located on the Central Experimental Farm of Agriculture and Agri-Food Canada in Ottawa. The soil was sampled from a depth of 5 to 20 cm in August 1993.

Enrichment of soil with <275 ppmv of CH_4. Enrichment cultures were made in nitrate mineral salts medium (NMS) (14) containing 3 nM Cu and 1 mM phosphate buffer at pH 6.0. Deionized distilled or twice-distilled water was used. Initially, 0.15 g of soil was added to 10 ml of NMS in 125-ml serum vials. The vials were capped with autoclaved butyl rubber stoppers, and CH_4 was added at a final gaseous mixing ratio of 75 ppmv. The enrichment cultures were incubated at 25°C. The CH_4 was replaced after declining to below 25 ppmv. After 7 months, and periodically thereafter for 4 years, subsamples of the enrichment culture were transferred into fresh medium. The CH_4 mixing ratio in the vials varied considerably during this enrichment period. It declined to 1 to 50 ppmv of CH_4 before being replaced but never exceeded 275 ppmv.

Some modifications were made during the enrichment period. Two years into the enrichments and thereafter, a pH 6.8 buffer and a 10-times-strength trace element solution (30 nM Cu) were used in the NMS medium. Since the butyl rubber stoppers often exuded inhibitory compounds after being autoclaved, the stoppers used after the initial transfer were sterilized by washing them in ethanol (50 to 80% [vol/vol]) followed by rinsing them three times in sterile distilled water.

All experiments described below were performed after the initial 4-year enrichment period. During this time, the culture had been diluted to >10^{20} times

* Corresponding author. Mailing address: Max-Planck-Institut für terrestrische Mikrobiologie, Karl-von-Frisch-Str., D-35043 Marburg, Germany. Phone: 49-6421-178-801. Fax: 49-6421-178-809. E-mail: conrad@mailer.uni-marburg.de.

TABLE 1. $K_{m(app)}$ values measured for methanotrophic bacteria, methanotrophic enrichment cultures, and soil[a]

Sample (reference)	$K_{m(app)}$ (μM CH_4)	
	<1 μM	>1 μM
Literature		
Methanotrophs, MMO (5, 18)		0.8–66
Aerobic interfaces of methanogenic soil (7, 39)		1.0–11
Aerobic upland soils (1, 3, 7, 8, 13)	0.010–0.280	
Aerobic upland soils, enriched with 20% CH_4 (1)	0.015–0.450	1.7–28
Organic soil (8)	0.060–0.280[b]	
Organic soil, enriched with 10% CH_4 (8a)		2.7–6.7
This study		
Enrichment culture		
Grown on <275 ppmv of CH_4	0.056, 0.083, 0.173, 0.186	
Transferred to 1,000 ppmv of CH_4	0.330, 0.566	
Transferred to 1% CH_4		1.96, 2.60
Coculture; type II methanotroph LR1 + *Variovorax* sp.	0.129, 0.177, 0.183, 0.188	

[a] For the literature, the range of reported values is shown. For experimental data, values from at least two trials (each with a separately grown culture) are shown.
[b] Estimated as 80 to 90 nM in the absence of NH_4^+.

the initial soil inoculum. Within this period a serial dilution series had been performed, and only the highest positive dilution (10^7 times) was maintained.

Kinetic experiments. Half-liter batches of the culture were grown on <200 ppmv of CH_4 for 1 to 4 months. Since our aim was to study substrate affinity rather than specific activity, cell counts were not done. However, from the dilution series described in the previous section, methanotroph densities of $<10^7$ cells ml^{-1} could be expected after such incubation times. Aliquots (2 to 8 ml, depending on the experiment) of the culture were transferred to 14-ml serum vials, and chloramphenicol at a final concentration of 50 μg $liter^{-1}$ was added to prevent further enzyme production. The vials were capped with butyl rubber stoppers and injected with CH_4 at mixing ratios ranging from 5 to 1,000 ppmv. The vials were incubated at 25°C and rotated around the short axis at 21 rotations per min. At 1- to 2-day intervals for 3 to 10 days, CH_4 was measured by injection of 0.3-ml gas samples into a Carlo Erba gas chromatograph equipped with a flame ionization detector (oven temperature, 100°C; injector temperature, 140°C; 3-m by 3-mm Porapak Q column).

Methane oxidation rates were estimated by linear regressions of CH_4 mixing ratios versus time. The regressions were effectively linear at CH_4 levels greater than the $K_{m(app)}$ (r^2, usually >0.95), proving that initial rates were being measured. The maximum decline of CH_4 over the incubations was always <50%. At each CH_4 concentration, blank vials containing only water were included to estimate CH_4 removal during gas chromatographic sampling and to correct the CH_4 oxidation rates in the culture. These corrected rates were plotted against the CH_4 levels at the time midpoint and fitted to a Michaelis-Menton hyperbolic model by using the least-squares iterative fitting procedure of Origin 4.1 (Microcal Software, Inc., Northampton, Maine).

Transfer of the enrichment culture into CH_4 at higher mixing ratios. The enrichment culture was inoculated into 50 ml of NMS in 125-ml serum vials and grown on CH_4 at initial mixing ratios of 1,000 ppmv and 1%. The CH_4 was replaced after declining to <20% of these initial values. The CH_4 was replaced five times for the 1,000-ppmv treatment and six times for the 1% treatment. Kinetic experiments were then performed as described above at a range of 20 to 3,000 ppmv of CH_4 (for the 1,000-ppmv enrichment culture, 1-ml aliquots were diluted to 2 ml and measured at 2-day intervals for 6 days; for the 1% enrichment culture, 1-ml aliquots were diluted to 6 ml total and measured at 1 or 2 intervals of 3 h).

Isolations. Members of the low-CH_4 enrichment culture (<275 ppmv of CH_4) were isolated by performing a decimal dilution series in liquid NMS medium and making spread plates of each dilution onto NMS medium solidified with 15 g of Bacto Agar (Difco Laboratories, Detroit, Mich.) $liter^{-1}$. The plates were incubated at 25°C in closed chambers containing a gaseous mixing ratio of 3% CH_4. Colonies which formed on plates of the highest two dilutions showing growth (10^5 to 10^6 times the initial inoculum) were restreaked for isolation (i) onto NMS solidified with Noble agar (Difco) and incubated under 3% CH_4 and (ii) onto plates of a general medium for culturing nonfastidious organisms, R2A agar (Difco), and incubated under air. Because we failed to isolate a methanotroph in this way (see Results), the procedure was repeated with a sample of the enrichment culture grown on 1% CH_4 (see the previous section).

DNA extraction. Extraction of DNA from the low-CH_4 enrichment culture (<275 ppmv of CH_4) and from the various isolates was adapted from the procedure of Moré et al. (28). Approximately 1 g of sterilized (170°C for 4 h) zirconia-silica beads of 0.1-mm diameter (Biospec Products Inc., Bartlesville, Okla.) was added to cell pellets in 2-ml screw-cap tubes. The cells and beads were suspended by vortexing them in 800 μl of NaH_2PO_4-Na_2HPO_4 buffer (120 mM; pH 8) and 260 μl of sodium dodecyl sulfate solution (10% [wt/vol] sodium dodecyl sulfate, 0.1 M NaCl, 0.5 M Tris-HCl, pH 8). The cells were lysed in a bead beater (Fastprep FP120; Savant Instruments Inc., Farmingdale, N.Y.) at a setting of 6.5 m s^{-1} for 45 s. The tubes were centrifuged for 3 min at 12,000 × *g*, and the supernatant was collected. The beads were then resuspended in 700 μl of NaH_2PO_4-Na_2HPO_4 buffer, and the DNA extraction was repeated. Proteins and debris were precipitated from the supernatant by adding 2.5 volumes of 7.5 M ammonium acetate, incubating the solution for 5 min on ice, and centrifuging it at 12,000 × *g* for 3 min. The DNA was precipitated by centrifugation (4°C; 45 min; 12,000 × *g*) with 70% (vol/vol) isopropanol added. The DNA pellet was washed with 70% (vol/vol) ethanol at 4°C, dried, and resuspended in 200 μl of elution buffer (Bio-Rad, Munich, Germany).

Comparative 16S rRNA gene sequence analysis. PCR-mediated amplification of the 16S rRNA genes from positions 28 to 1491 (numbering according to the International Union of Biochemistry nomenclature for *Escherichia coli* 16S rRNA) and sequencing analyses were done as previously described (22). Phylogenetic placement of strain LR1 was performed with the ARB program package (37). The 16S ribosomal DNA (rDNA) sequence of strain LR1 was integrated into a database of about 6,000 complete or partial bacterial 16S rRNA sequences (24, 31, 38) with the automatic alignment tool of the ARB program package. This procedure showed strain LR1 to be a member of the alpha subclass of the class *Proteobacteria*. The phylogenetic position of strain LR1 was determined in more detail by comparing its 16S rDNA gene sequence with alpha proteobacterial reference sequences. The tree topology was evaluated by a distance matrix analysis. For phylogenetic inference, the only nucleotide sequence positions considered were those which contained identical nucleotides in at least 50% of a representative selection of 16S rRNA sequences from the major lineages of the alpha subclass of proteobacteria (1,353 nucleotide sequence positions). Evolutionary distance values between pairs of microorganisms were calculated with the Felsenstein correction (ARB) (11). The tree was constructed by the neighbor-joining algorithm (33). The statistical significance values of interior branch points were tested in a bootstrap analysis by the neighbor-joining method (ARB; 1,000 data resamplings).

Comparative analysis of *pmoA* and *mxaF* gene fragments. (i) PCR amplification. Primers targeting gene fragments of *pmoA* (A189f and A682r) (16), coding for a subunit of particulate MMO (pMMO), and of *mxaF* (*mxaF* f1003 and *mxaF* r1561) (25, 27), coding for the large subunit of methanol dehydrogenase, were used for PCR amplification. A GC clamp (CCCCCCCCCCCCCCGCCCCCCGC CCCCCGCCCCCGCCGCCC) was attached to the 5′ end of each forward primer. PCR was performed in 50-μl reaction mixtures with an Eppendorf (Hamburg, Germany) gradient cycler. *Taq* polymerase (Perkin-Elmer Applied Biosystems, Branchburg, N.J.) was added to 0.5 μM concentrations of each primer and a PCR premix (Epicentre Technologies, Madison, Wis.). The *mxaF* fragment was amplified as described previously (27). For the *pmoA* amplification a touchdown program was developed, consisting of an initial denaturing step (94°C; 3 min) followed by 20 touchdown cycles (62 to 52°C), eight further cycles (52°C for 1 min followed by 72°C for 45 s), and a final extension (72°C; 5 min). The PCR products were analyzed on 3% agarose gels stained with ethidium bromide.

(ii) DGGE. PCR products were separated on 1-mm-thick polyacrylamide gels (6.5% [wt/vol] 37.5:5 acrylamide-bisacrylamide) (Bio-Rad) poured on GelBond support medium (FMC Bio Products, Rockland, Maine). They were prepared and run in Tris-acetic acid-EDTA buffer (0.04 M Tris-base, 0.02 M sodium acetate, 1 mM EDTA) at pH 7.4 and 60°C. The Dcode electrophoresis system (Bio-Rad) was used for separation. Denaturing gradient gel electrophoresis (DGGE) conditions for the various PCR products were optimized by perpen-

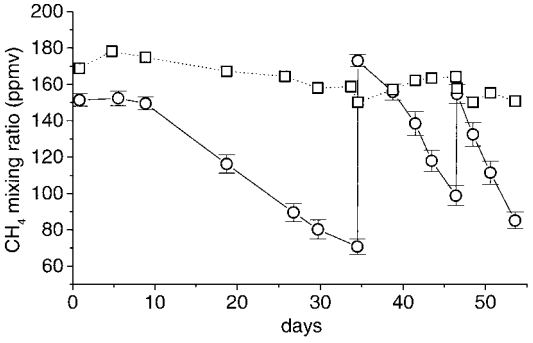

FIG. 1. Oxidation of CH_4 at mixing ratios of <200 ppmv (○) in closed vials (125 ml) containing 50 ml of NMS inoculated with the enrichment culture at day 0. Methane was added to the vials at 34 and 46 days. The data are the means of results with six vials ± 1 standard error of the mean. □, mean of results with three uninoculated vials.

dicular DGGE. For the *pmoA* fragments, a gradient of 35 to 80% denaturant (80% corresponded to 6.5% [vol/vol] acrylamide, 5.6 M urea, and 32% [vol/vol] deionized formamide) at a constant voltage of 200 V for 6 h was used. For the *mxaF* fragments, a gradient of 20 to 70% denaturant at a constant voltage of 150 V for 5 h was used. The gels were stained with 1:50,000 (vol/vol) SYBR-green I (Biozym, Hessisch-Oldendorf, Germany) for 30 min and then scanned on a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

(iii) DNA sequencing and construction of *pmoA*- and *mxaF*-based trees. Distinct bands were excised from the SYBR-green-stained gels with sterile pipette tips and suspended in 200 μl of H_2O. The bands were reamplified and rerun on DGGE to ensure purity.

The PCR products from the excised bands were purified with the Easy-Pure DNA purification kit (Biozym). The concentration and purity of the PCR products were determined by absorption at 260 and 280 nm of a 1:20 dilution. The sequencing reactions were performed in both directions with the PRISM dye terminator cycle-sequencing kit (Perkin-Elmer Applied Biosystems). Products of the cycle-sequencing reaction were purified from excess dye terminators and primers with Microspin G-50 columns (Pharmacia, Uppsala, Sweden) and sequenced on an automatic DNA sequencer (model 373A; Perkin-Elmer Applied Biosystems). Phylogenetic trees were constructed with the cluster alignment algorithm of the DNA-Star software package (Lasergene Inc., Madison, Wis.).

Comparative sequence analysis of *mmoX*. A gene fragment of *mmoX* coding for the alpha subunit of soluble MMO (sMMO) was amplified from both the low-CH_4 enrichment culture (<275 ppmv of CH_4) and the methanotrophic isolate LR1 with primers (*mmoX* f882 and *mmoX* r1403) under PCR conditions described previously (25). The resulting PCR products were checked for size and purity on a 1.5% agarose gel and purified with the Prep-A-Gene system (Bio-Rad). Sequencing was performed as described above.

Nucleotide sequence accession numbers. The nucleotide sequences of the nearly complete 16S rRNA gene and of partial *mmoX*, *mxaF*, and *pmoA* genes and gene fragments of strain LR1 have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. Y18442, Y18440, Y18441, and Y18443, respectively.

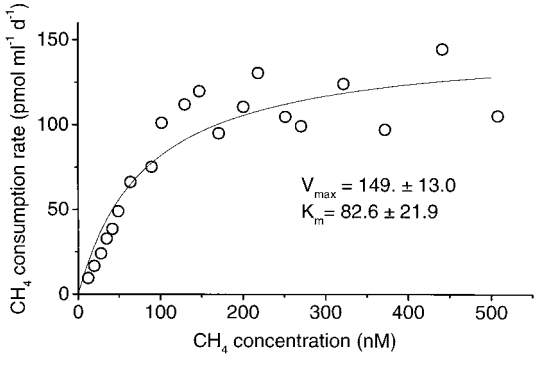

FIG. 2. Kinetic curve of CH_4 oxidation in a soil enrichment culture which was grown continuously on CH_4 at a gaseous mixing ratio of <275 ppmv. Each symbol represents the mean of results with two samples.

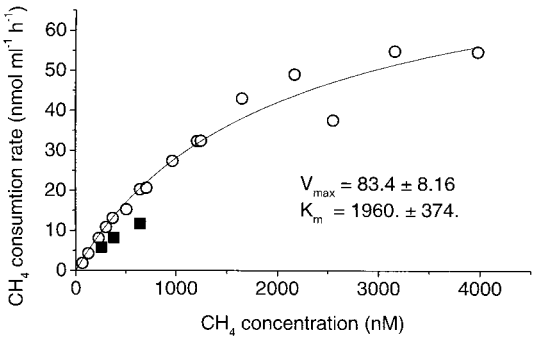

FIG. 3. Kinetic curve of CH_4 oxidation in a soil enrichment culture initially grown on CH_4 at a gaseous mixing ratio of <275 ppmv and later on 1% CH_4. Each symbol represents the mean of results with two samples. ■, samples diluted 1:1 with water.

RESULTS

Soil enrichment cultures grown on <275 ppmv of CH_4. Soil enrichment cultures grown on 10 to 20% CH_4 display low-affinity kinetics [$K_{m(app)} > 1$ μM (Table 1)]. We therefore chose a lower CH_4 mixing ratio in an attempt to enrich for higher-affinity methanotrophic activity.

The CH_4 oxidation rate of soil inoculated into NMS showed a gradual increase with time when the soil was incubated at CH_4 mixing ratios of as little as 75 ppmv. An enrichment culture was obtained by continuous growth on <275 ppmv of CH_4 for 4 years. The increase in activity was very slow, typically requiring a week or more to double. An example showing the increasing rate of CH_4 oxidation over time in the enrichment culture is shown in Fig. 1. Blank vials containing uninoculated medium plus CH_4, which were sampled in exactly the same way as the enrichment cultures, never developed methanotrophic activity. Therefore, contamination of the cultures with a methanotroph from another source was unlikely.

Kinetics. A typical kinetic curve from the low-CH_4 enrichment culture is shown in Fig. 2. The measured $K_{m(app)}$ in four trials varied from 56 to 186 nM CH_4 (40- to 132-ppmv mixing ratio) (Table 1). This range overlaps the $K_{m(app)}$ estimated for the organic soil itself (Table 1).

When samples of the enrichment were transferred to new medium and grown on CH_4 at higher mixing ratios, the $K_{m(app)}$ values increased (Table 1). Growth on 1,000 ppmv of CH_4 resulted in $K_{m(app)}$ values higher than those in the initial low-CH_4 enrichment (<275 ppmv of CH_4) but still lower than those in pure methanotroph cultures. Enrichments grown on 1% CH_4 had $K_{m(app)}$ values typical for methanotrophic cultures (Table 1). A kinetic curve for a culture grown on 1% CH_4 is shown in Fig. 3. The high $K_{m(app)}$ value was not a result of phase transfer limitation or poor mixing of CH_4, as demonstrated by the fact that diluting the culture 1:1 with water resulted in about a 50% reduction of the CH_4 oxidation rate (Fig. 3).

Members of the methanotrophic enrichment culture. When samples of the low-CH_4 enrichment culture (<275 ppmv of CH_4) were plated onto NMS agar, various types of colonies grew. These had sizes and morphologies typical of heterotrophic organisms which grow on trace contaminants in the medium (14). Four separate organisms which grew when transferred to complex medium (R2A agar) were isolated. These were identified based on partial 16S rRNA gene sequences. The closest relatives of these isolates were "*Pseudomonas pavonacea*" IAM1155 (similarity, 99.0% based on an analyzed

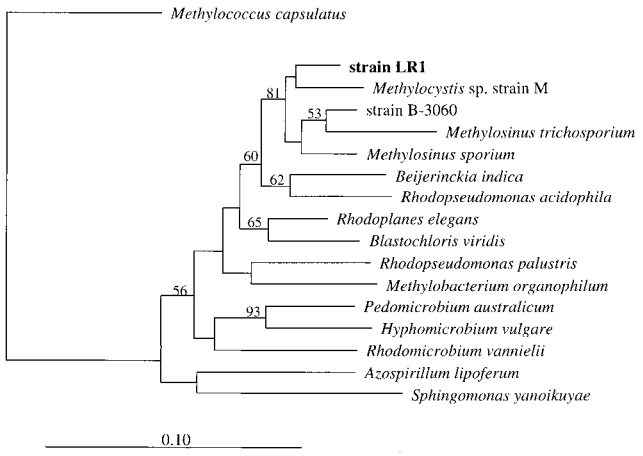

FIG. 4. Phylogenetic tree, based on 16S rRNA gene sequences, showing the relationship of isolate LR1 to other type II methanotrophs of the *Methylocystis*/*Methylosinus* group and to representative members of the alpha subclass of the class *Proteobacteria*. The 16S rRNA gene sequence from *Methylococcus capsulatus* was used as an outgroup reference. The numbers indicate the bootstrap values (percentage of outcome) for the respective interior branch points from a neighbor-joining test. Only values above a threshold of 50% are shown. The scale bar represents the estimated number of base changes per nucleotide sequence position.

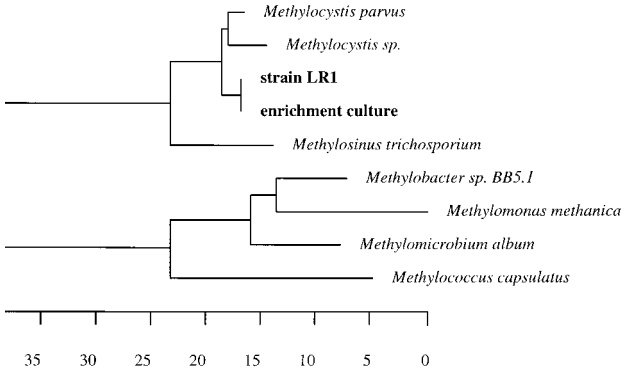

FIG. 5. Unrooted phylogenetic tree, based on derived amino acid sequences of *pmoA* gene fragments, showing the identity of the *pmoA* product from isolate LR1 and that from the high-affinity enrichment culture (grown on <275 ppmv of CH_4) and their relationship to other methanotrophic species. The distance bar represents percent dissimilarity.

stretch corresponding to *E. coli* 16S rRNA numbering positions 104 to 1293), *Variovorax paradoxus* (similarity, 99.2%; numbering positions 158 to 1192), *Bradyrhizobium elkanii* USDA76 (similarity, 98.5%; numbering positions 28 to 819), and *Hyphomicrobium vulgare* MC-750 (similarity, 96.9%; numbering positions 28 to 908).

None of these isolates consumed CH_4 in pure culture, and none showed a *pmoA* gene product (data not shown). However, transfer of the low-CH_4 (<275 ppmv of CH_4) liquid enrichment culture to an atmosphere containing 1% CH_4 resulted in a turbid culture which did produce thick methanotroph colonies (14) when streaked onto NMS agar and grown on 3% CH_4. Isolated colonies of a methanotroph designated LR1 were selected and stored in liquid NMS on 1% CH_4.

Gene primers for *mmoX* (coding for the alpha subunit of sMMO) and *pmoA* (coding for a subunit of pMMO) both gave a signal from isolate LR1. The membership of strain LR1 within the type II methanotrophs of the *Methylocystis*/*Methylosinus* cluster in the alpha subclass of the class *Proteobacteria* was clearly shown by comparative analysis of its 16S rRNA (Fig. 4), its *pmoA* (Fig. 5), and its *mxaF* (Fig. 6) gene sequences.

DGGE analyses. The results of the DGGE analyses suggested that there was a single methanotrophic species present in the low-CH_4 enrichment culture (<275 ppmv of CH_4). The *pmoA* primer system, which should detect all known methanotrophic species, produced a single band on the DGGE for the enrichment (Fig. 7). Although methanotrophs appear to contain two copies of *pmoA* (36), these are not always distinguishable on DGGE. Two *pmoA* bands were occasionally evident from methanotroph isolates when DGGE gels were silver stained, but only a single band was evident from the enrichment culture and from isolate LR1 (data not shown).

The *mxaF* primer system also produced only one clear band (Fig. 7). A second faint band may have been present in the *mxaF* gel, but is migration pattern corresponded to that of a methylotrophic *Hyphomicrobium* sp. isolated from the culture.

The sequences of the *pmoA* (Fig. 5), *mxaF* (Fig. 6), and *mmoX* (data not shown) PCR products retrieved from the low-CH_4 enrichment (grown on <275 ppmv of CH_4) were all identical to those of the respective PCR products retrieved from isolate LR1.

Isolate LR1 was therefore the only detectable methanotroph in the enrichment culture grown on <275 ppmv of CH_4. The data cannot completely rule out the possibility that another methanotroph was present, perhaps in much lower abundance, but its *mxaF*, *pmoA*, or *mmoX* genes were not detected. They also cannot rule out the possibility that an organism without either *mxaF*, *pmoA*, or *mmoX* was responsible for the high-affinity CH_4 oxidation. Unidentified species were present in the enrichment, as shown by a DGGE performed with a universal 16S rDNA primer system (data not shown). In this gel, several bands were visible which did not have the same migration patterns as any of the five isolated organisms described above.

Isolate LR1 was therefore grown in a coculture with the *Variovorax* sp. isolated from the enrichment. The *Variovorax* was included because it stimulated the growth of LR1, although alone it could not consume CH_4 (data not shown). This coculture was grown on <275 ppmv of CH_4 for 4 to 5 months,

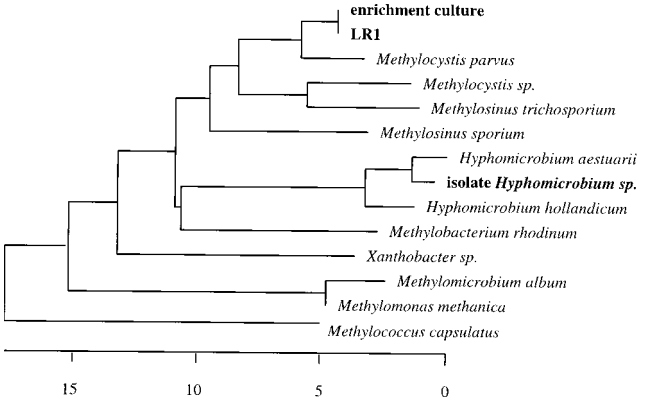

FIG. 6. Unrooted phylogenetic tree, based on derived amino acid sequences of *mxaF* gene fragments, showing the identity of the *mxaF* product from isolate LR1 and that from the high-affinity enrichment culture (grown on <275 ppmv of CH_4) and their relationship to other methylotrophic species. The distance bar represents percent dissimilarity.

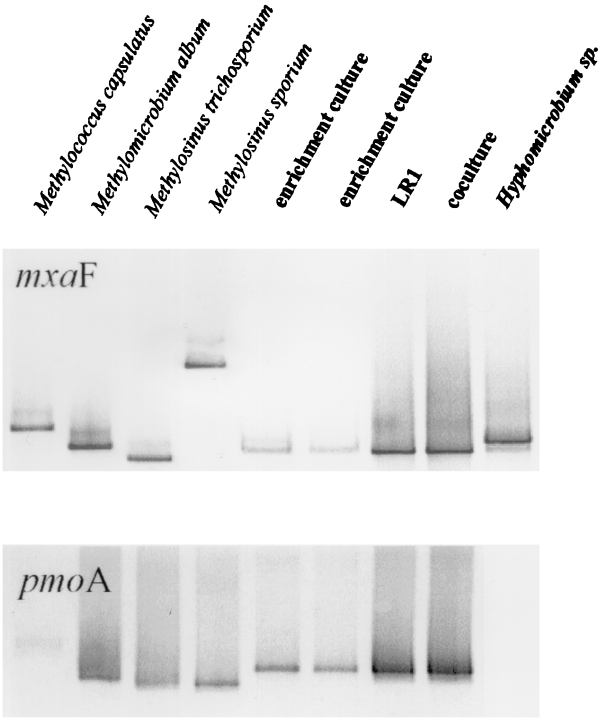

FIG. 7. DGGE patterns of DNA, amplified with primers for *mxaF* and *pmoA*, from selected methanotrophic strains (first four lanes), the high-affinity enrichment culture (grown on <275 ppmv of CH_4), the methanotrophic isolate LR1, a coculture of LR1 with a *Variovorax* sp., and a *Hyphomicrobium* sp. isolated from the enrichment culture.

and kinetic curves were measured as previously described. A $K_{m(app)}$ value of 129 to 188 nM was estimated in this coculture (Table 1). This range overlaps that of the more complex enrichment culture and is considerably lower than any range previously reported for methanotrophic bacteria. Isolate LR1 was therefore probably the high-affinity CH_4 oxidizer in the initial, low-CH_4 enrichment culture (<275 ppmv of CH_4).

DISCUSSION

Methanotrophs in soils exposed to only atmospheric CH_4 possess a higher affinity for CH_4 than do pure cultures of methanotrophic bacteria, which are routinely grown on CH_4 at mixing ratios of 10 to 20% (Table 1). There is no conclusive evidence indicating whether this high-affinity activity is carried out by novel organisms or simply by known methanotrophs behaving in an unknown manner. Recently it was shown that the $K_{m(app)}$ (CH_4) in *M. albus* varied depending on whether the organisms were grown on CH_4 or methanol as an energy source (4). This demonstrates that the $K_{m(app)}$ in known methanotrophic bacteria changes with culture conditions, although neither reported value was near the low-nanomolar $K_{m(app)}$ values measured in upland soil (both were >1 μM) (4). The $K_{m(app)}$ of soils enriched with CH_4 at high mixing ratios has also been shown to vary with the CH_4 supply, but again only in the $K_{m(app)}$ range of >1 μM (19).

Our data suggest two major points: that a type II methanotrophic species in mixed culture can exhibit $K_{m(app)}$ values which vary depending on the CH_4 supply and that the $K_{m(app)}$ of such a culture can approach that measured in upland soils when low CH_4 mixing ratios (<275 ppmv) are used for growth. We produced a methanotrophic enrichment culture which, based on its *pmoA*, *mmoX*, and *mxaF* products, contained a single detectable methanotroph. This organism (LR1) was a type II species of the *Methylosinus*/*Methylocystis* group. In the enrichment culture, and in a coculture containing only LR1 and a *Variovorax* sp., growth on <275 ppmv of CH_4 resulted in $K_{m(app)}$ values of 56 to 188 nM. However, when the CH_4 mixing ratio was raised to 1%, the $K_{m(app)}$ shifted upward to a value typical for methanotrophic isolates (>1 μM [Table 1]).

The organic soil from which our enrichment cultures were made consumes atmospheric CH_4 over much of the year (9). We previously measured $K_{m(app)}$ values of 60 to 280 nM CH_4 in this soil, estimating the value in the absence of inhibitory NH_4^+ as 80 to 90 nM (8). Because the $K_{m(app)}$ values in our cultures grown on <275 ppmv of CH_4 were in the same range as these values, species such as LR1 could be the active methane oxidizers in this soil. It is therefore unnecessary here to postulate novel organisms as the agents of atmospheric-methane oxidation. The 16S rRNA gene sequence, as well as sequences for the functional genes *pmoA* and *mxaF*, which are also useful phylogenetic markers for methanotrophic bacteria (26, 27), all showed that LR1 clusters with other type II species and does not belong to a novel lineage of methanotrophs.

The $K_{m(app)}$ values in our cultures grown on <275 ppmv of CH_4 were lower than the lowest previously reported value for methanotrophic isolates. However, the values in our cultures, and in the organic soil itself, are higher than those measured in many other upland soils. These can be as low as 10 nM (1, 3, 13). This organic soil is periodically methanogenic (9) and possibly supports a methanotrophic flora different from that of other soils. Although organisms such as LR1 may account for atmospheric-CH_4 oxidation in this organic soil, other species may dominate in other soils. On the other hand, the fact that the $K_{m(app)}$ in our cultures was variable suggests that even lower values may be inducible under more oligotrophic conditions.

The CH_4 concentration was a key factor in determining $K_{m(app)}$, but this could be mediated through the MMO enzyme, the methanotroph, or the bacterial community as a whole. Only apparent kinetic coefficients are measured in a study with a mixed microbial community, and it cannot be assumed that these represent true enzyme properties. A variety of reasons could therefore account for the variable $K_{m(app)}$. We propose three possibilities. (i) A modified form of MMO is responsible for high-affinity activity. (ii) The measured $K_{m(app)}$ is diffusion limited and affected by physiological properties of methanotrophs which change with growth conditions. Although the specific CH_4 uptake rate in methanotrophs (10^{-15} mol of CH_4 $cell^{-1}$ h^{-1}) (15) is 100 to 1,000 times lower than the maximum rate of CH_4 diffusion through the cell boundary layer (calculated with the van Smoluchowski equation [21]), diffusion limitation could occur if cells aggregate or if the cell envelope presents a diffusion barrier (21). In a diffusion-limited system the measured $K_{m(app)}$ values are affected by the aggregation, the specific activity, or the envelope structure of the cells. (iii) It is difficult to apply simple Michaelis-Menton kinetics to a complex terreactant enzyme such as MMO. The $K_{m(app)}$ for a substrate is a function of both an equilibrium constant (binding of substrate to enzyme) and a reaction rate constant (decomposition to product). These may be altered by the availability of cosubstrates. For example, it might be expected that the cosubstrate NADH is more limiting in methanotrophic cultures grown on <275 ppmv of CH_4 than in those grown on 1% CH_4. If NADH limitation slowed the decomposition of the MMO-CH_4 complex (although this does not agree with the present model of the sMMO reaction sequence [23]), a lower $K_{m(app)}$ could result (35).

Methane oxidation rates in soil have been stimulated by incubation on >1,000 ppmv of CH_4 (1, 2, 29, 34). Transient or minor increases may follow incubation with less CH_4 (3, 12), but until now no study had produced a long-term methanotrophic enrichment with <300 ppmv of CH_4 (2, 4, 20, 34). It has been suggested that periodic methanogenic events (9) or alternate substrates, such as methanol (4, 17), are necessary for growth and maintenance of the methanotrophs which consume atmospheric CH_4 in soils. The results of the present study demonstrate that methanotrophs are more oligotrophic than previously believed but they do not contradict these theories. Our data suggest that the $K_{m(app)}$ value of known methanotrophic species is dependent on environmental conditions, especially the CH_4 supply, and therefore that the low $K_{m(app)}$ values measured in upland soils do not necessarily imply that novel species of "atmospheric-methane oxidizers" exist.

In summary, we propose that organisms closely related to known type II methanotrophic species contribute to high-affinity atmospheric CH_4 consumption in an organic soil and that the $K_{m(app)}$ value of methanotrophic organisms is determined by the conditions under which they are cultured, especially by the CH_4 supply.

ACKNOWLEDGMENTS

P.F.D. was supported by stipends from the Max-Planck Gesellschaft (Germany) and Eco-Research Council (Canada). Portions of the investigation were supported by grants from the EC RTD Programme Biotechnology (contract BIO4-CT96-0419) and the Natural Sciences and Engineering Research Council of Canada (contract GP3252 to R.K.).

We thank Thomas Lukow and Sonja Fleissner for technical assistance.

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Lebenslauf

Thilo Hans Eckhardt Henckel geboren am 11.04.1970 in Hannover.

Schulausbildung

08.1976-08.1982	Grundschule / Orientierungsstufe
09.1982-07.1984	Neues Gymnasium Stadthagen
09.1984-07.1987	Morris Hills Highschool, Rockaway, N.J., USA
08.1987-05.1989	Neues Gymnasium Stadthagen
05.1989	Allgemeine Hochschulreife

10.1989-09.1991	Bundeswehr

Studium

10.1991-06.1997	Studium an der Georg-August-Universität Göttingen
10.1993	Vordiplom
01.1996-12.1996	externe Diplomarbeit am Max-Planck-Institut für terrestrische Mikrobiologie, Marburg, unter Anleitung von Prof. R. Conrad "Mikrobielle Produktion von Stickoxiden und Oxidation von Methan in belüfteten Reisfeldböden"
02. 1997	Diplom
seit 05. 1997	Dissertation am Max-Planck-Institut für terrestrische Mikrobiologie, Marburg unter Anleitung von Prof. R. Conrad

Wissenschaftliche Publikationen:

Henckel, T. and Conrad R. (1998) Characterization of microbial NO production, N_2O production and CH_4 oxidation initiated by aeration of anoxic rice field soil, *Biogeochemistry*, 40, 17-36.

Dunfield, P., F., Liesack, W., Henckel, T., Knowles, R., Conrad, R. (1999) High-affinity methane oxidation by a soil enrichment culture containing a type II methanotroph, *Appl. Environ. Microbiol.*, 65, 1009-1014.

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Bodelier, P. L. E., Roslev, P., Henckel, T., and Frenzel, P. (2000) Stimulation by ammonium-based fertilisers of methane oxidation in soil around rice roots, *Nature*, 403, 421-424.

Henckel, T., Jäckel, U., Schnell, S., and Conrad, R. (2000) Molecular analyses of novel methanotrophic communities in forest soil that oxidize atmospheric methane, *Appl. Environ. Microbiol.*, 66, 1801-1808.

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Henckel, T., Jäckel, U., and Conrad, R. (submitted) Vertical distribution of the methanotrophic comunity after drainage of rice field soil. *Fems Microbiology Ecology*

Danksagung

Meinem Doktorvater Herrn Prof. Dr. Ralf Conrad möchte ich für seine Begleitung, die Anregungen und stete Diskussionsbereitschaft ausdrücklich danken. Er hat mir großen Forschungsfreiraum gewährt und meinem Arbeiten Vertrauen entgegengebracht. Beides hat es mir leicht gemacht meine Promotion nach meinen Vorstellungen und mit viel Freude zu verwirklichen.

Herrn Prof. Dr. R. Thauer möchte ich für die Begutachtung der Arbeit danken.

Herzlichen Dank an Dr. Paul Bodelier, Dr. Peter Dunfield, PD. Dr. Peter Frenzel und Udo Jäckel für die schöne und produktive Zusammenarbeit, für viele Anregungen und Diskussionen über die Methanoxidation, sowie vielfältigste Unterstützung meiner Arbeit.

Dr. Michael Friedrich möchte ich für die Einführung in die DGGE danken, für technische Unterstützung und Zusammenarbeit.

Für die PLFA-Analysen danke ich Dr. Peter Roslev, Aalborg, D.K., sowie Dr. Hans Scholten für die Produktion des $^{14}CH_4$.

Bianca Wagner sei gedankt für die vielen und immer unkomplizierten Hilfen im Labor, beim Sequenzieren und Klonieren. Ich habe ihre Kompetenz bei allen molekularbiologischen Fragestellungen gerne in Anspruch genommen.

Sonja Fleissner danke ich für so manches Sequenziergel.

Ganz besonders danke ich Dr. Manigée Derakshani für die Korrektur und die hilfreichen Anmerkungen zu meiner Dissertationsschrift, sowie Heike und Ulrike Henckel für die germanistische Schadensbegrenzung.

Der gesamten Abteilung Conrad gilt mein Dankeschön für das gute und freundliche Klima, besonders aber Markus Drescher für die ständige Bereitschaft zur Behebung meiner Computerprobleme,

und Dorothee Geißlinger für den Beistand, die Unterstützung und mein Ertragen nach schlecht gelaufenen Versuchen.

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig verfasst, keine anderen als die angegebenen Hilfsmittel verwendet und sämtliche Stellen, die im Wortlaut oder dem Sinn nach anderen Werken entnommen sind, mit Quellenangaben kenntlich gemacht habe. Die Versicherung schließt Zeichnungen und Skizzen mit ein.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.